# CANCER TREATMENT REPORTS

## Laboratory Models and Clinical Cancer

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# CANCER
## TREATMENT REPORTS

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**9.** The methods of statistical analysis should be described in detail sufficient that a knowledgeable reader could reproduce the analysis if the data were available.

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* *From* Methodologic Guidelines for Reports of Clinical Trials (Cancer Treat Rep 69:1–3, 1985).

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# Laboratory Models and Clinical Cancer

Editors:
Robert E. Wittes
Abraham Goldin

Frank M. Schabel, Jr (1918–1983)

# INTRODUCTION

This issue of *Cancer Treatment Reports* is dedicated to Dr. Frank M. Schabel, Jr. With Dr. Howard Skipper and other colleagues at the Southern Research Institute, Frank Schabel devoted his professional life to the study of chemotherapy in model tumor systems. His research group was instrumental in developing general principles of treatment that have exerted enormous influence on the course of therapeutic studies in human cancer.

At a time when scientific developments have never held greater promise for the development of effective therapies, it seems appropriate to consider certain aspects of the critical interface between laboratory and clinic. The papers included in this issue deal with selected areas in which cross-fertilization between laboratory and clinic have been or promise to be particularly noteworthy. Appropriately for an issue dedicated to Frank Schabel, most of the contributions deal with various attempts to optimize chemotherapy; these include the identification of more active drugs, improvements in preclinical development that will permit more accurate prediction of clinical effects, and the more effective use of drugs in combination with each other and with other modalities. In addition to traditional cytotoxic approaches, modification of host response to tumor, induction of tumor cell maturation, and interference with the process of metastasis will receive increasing emphasis in coming years.

Although not a clinician himself, Schabel reveals in his writings a continuing and deep concern with the implications of his work for the treatment of human cancer. This abiding concern is necessary in any successful effort to bridge the gap between experimental or theoretical models and clinical realities. As our understanding of the basic biological processes underlying the malignant state increases, we hope that Frank Schabel's work will serve as a lasting example to all who attempt to apply results obtained in model systems to the clinic.

Robert E. Wittes
Abraham Goldin

# Laboratory Models: Some Historical Perspective

**Howard E. Skipper**[1,*]

The key word in the title of this rather informal paper is perspective—as viewed from my own vantage point and colored by my own experiences. No attempt will be made to recount all of the important contributions to the theory and practice of cancer treatment that have been made by (*a*) those who have used experimental models *wisely*, and (*b*) those who have successfully translated *in both directions* between experimental and clinical observations.[2]

By laboratory or experimental models, I mean all sorts of things: experimental leukemias, lymphomas and solid tumors in vivo, human and animal cancer cells in culture, bacterial cells in culture, appropriate biochemical systems, and, for goodness' sake, let's not exclude mathematical models that help us to test the reasonableness of our theories and interpretations of large bodies of experimental and clinical data.[3]

By using models *wisely*, I mean:

(*a*) posing realistic questions that have to do with treatment of disseminated cancers and why systemic treatment succeeds when it succeeds and fails when it fails;

(*b*) choosing a model or models well suited to gaining reliable information regarding a particular question;

(*c*) designing experiments in a manner likely to provide a definitive answer rather than data that might be interpreted in many ways; and

(*d*) interpreting experimental (and clinical) results with care, considering strengths and weaknesses, alternative interpretations, and compatibility, or lack of compatibility, with reasonable theory and diverse "hard data."

By translation between experimental and clinical observations, I mean, first, recognition that certain therapeutic principles observed in an experimental model may apply to a human cancer, or many human cancers, and, second, that carry-over is unlikely unless one takes into consideration very significant *quantitative differences* between the model and a human cancer and between different human cancers.

By quantitative differences, I mean differences in tumor burden and the relative proportion of limiting T/R cells[4] in neoplasms of the same and different sizes, differences in proliferation and growth rates, and pharmacokinetic differences between small animals and small and large people.

I assume that most investigators are aware that both experimental neoplasms in vivo and cell culture systems have been used in the past for "screening." Some time ago I retired from debates having to do with which is the best in vivo or in vitro model or models for screening, but not without some personal opinions that I won't belabor. They have to do with using model systems *wisely* and taking into consideration quantitative differences between models and human cancers.

I have long believed in both in vivo and in vitro screening. A research benefit we derived over the years from doing in vivo screening was that we usually had a variety of experimental neoplasms on hand and people who understood their behavior and responsiveness to different treatments and knew how to design reasonably quantitative multiarmed trials with single drugs and combinations of drugs used alone or in an adjuvant setting. This allowed us to plan and carry out nonroutine experimental therapeutic research on questions that puzzled us, our sponsors, and clinical associates in many institutions. Sometimes I think that our research in different areas of experimental therapeutics has been as useful as the screening we have done, but I doubt that we could have done one very well without the other.

A benefit we derived from in vitro screening (in cell culture systems and biochemical systems) was that questions that were difficult, or almost impossible, to answer in in vivo systems sometimes could be answered rather easily in in vitro systems.

[1]Southern Research Institute, Birmingham, AL.

[2]Since this issue of *Cancer Treatment Reports* is in honor of Frank M. Schabel, Jr, with whom I worked on a day-to-day basis for over 30 years, I would like the privilege of placing his name high on the list of scientists who used experimental models *wisely* in attempting to gain information that would be useful in designing treatment of disseminated human cancers.

[3]Cross-disciplinary confirmation is comforting, but interpretations that require repealing the laws of thermodynamics or time-honored mathematics laws are distressing.

[4]*See* figure 1 for definitions of tumor stem cell types that are of major concern in cancer chemotherapy, and figure 2 for idealized time-action curves of types and shapes observed at both the experimental and clinical levels.

[*]*Reprint requests to:* Dr. Howard E. Skipper, Southern Research Institute, PO Box 55305, Birmingham, AL 35255-5305.

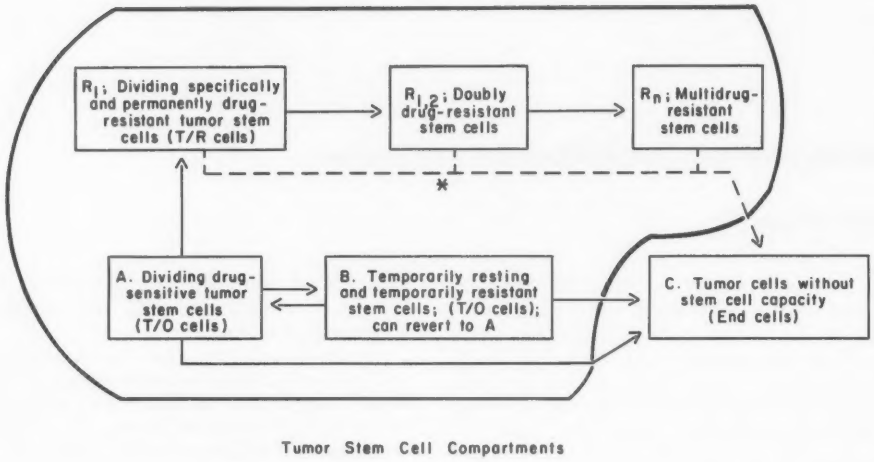
$R_1$; Dividing specifically and permanently drug-resistant tumor stem cells (T/R cells)
$R_{1,2}$; Doubly drug-resistant stem cells
$R_n$; Multidrug-resistant stem cells
*
A. Dividing drug-sensitive tumor stem cells (T/O cells)
B. Temporarily resting and temporarily resistant stem cells; (T/O cells); can revert to A
C. Tumor cells without stem cell capacity (End cells)
Tumor Stem Cell Compartments
* Resistant phenotypes also may go into and out of resting phase but they remain T/R cells.

FIGURE 1.—Tumor stem cell heterogeneity and responsiveness to chemotherapy. The origin of different tumor stem cell types is presumed to be T/0 → $T/R_1$ → $T/R_{1,2}$ → $T/R_n$. Before treatment, one may expect the following proportions of tumor stem cell types in any type of neoplasm: T/0 > $T/R_1$ > $T/R_{1,2}$ > $T/R_n$ (if any). Before treatment, the ratio of T/R to T/0 cells will almost never be as high as 1.0. Chemotherapeutic cure requires the eradication of all tumor stem cells, regardless of type. There is an invariable inverse relation between tumor burden and curability by chemotherapy, albeit with a wide variation in total burden curable in different cancers. One may expect a direct relationship between the total tumor burden and the ratio of T/R to T/0 cells as well as the ratio of resting to dividing T/0 cells. Chemotherapeutic selection of T/R cells (increasing the T/R to T/0 ratio) will account for objective tumor response followed by tumor progression during continuing treatment with the same drug or drugs; selection of resting T/0 cells will not.

As I have witnessed the regular cyclical changes in "majority opinion" regarding screening models (that have taken place between 1945 and 1984), I often have been reminded of this quotation: "Those who cannot remember history are doomed to repeat it."

Having indicated what I mean by certain words in the first paragraph of this general introduction, and what I can't do in this rather short paper, I might as well admit what I was asked to do by the editors.

"We should like to invite you to contribute an article which would lend some historical perspective to experimental therapeutics in murine models."

"What have the major lessons been and what has been the intellectual process by which the lessons were learned?"

"In what ways did lessons learned in the clinic feed back to the laboratory?"

On the following pages I will reminisce and ramble a bit and perhaps (now and then) try to keep in mind what I was asked to do. To be frank, it isn't easy to recall what I thought 10, 20, 30, or 40 years ago—much less what I thought other people thought. It is even more difficult to be confident regarding the intellectual process by which lessons learned at the experimental and clinical levels began to overlap, mesh, and influence both experimental and clinical research on cancer treatment.

## The Early Days (1945–1955)

To say that most of us who began looking for more effective means for treating disseminated cancers in the mid-1940s were a bit naive is an understatement. Those who had worked with alkylating agents during World War II were well aware of their leukopenic action in mice and man. We also knew of several patients with advanced lymphomas who, after treatment with mechlorethamine, exhibited dramatic but temporary remissions. In addition, minimum responses of murine and human leukemia to benzene, potassium arsenite, and urethan had been reported.

But the real motivation in the early days came from dreams of effective treatment of disseminated malignancies that could not be cured by surgery or ionizing radiation. The then recent successes of chemotherapy in treating disseminated bacterial diseases and malaria were our models, despite proclamations that this was a foolish notion. In retrospect, cancer chemotherapy in the early days was no place for a born pessimist, but it was alright for young enthusiasts so long as they were not zealots with no respect for documentation. What were the early working hypotheses? For good reason, they often were not enunciated in any detail.

It may be of interest to recall that in vitro bacterial

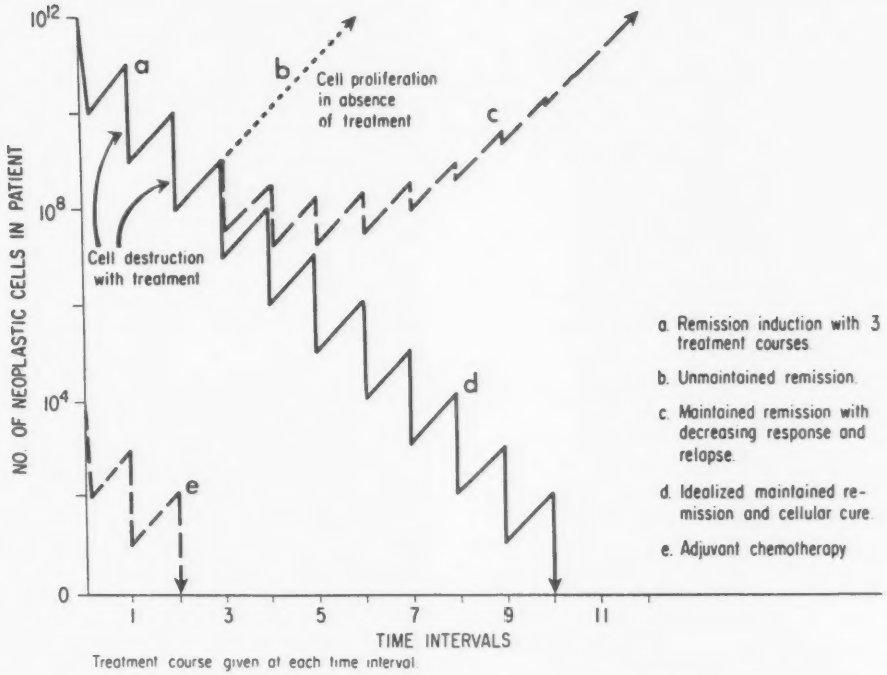


FIGURE 2.—Idealized time-action response curves for total tumor stem cells (*from* Blum RH, Frei E, III, and Holland JF. Cancer Medicine, 2nd ed. Philadelphia, Lea and Febiger, 1982, pp 730-752). ***Curve ab***: cessation of treatment after 3 courses. A second response with the same treatment will depend on the T/R to T/0 stem cell ratio at the cessation of treatment and a relapse. ***Curve ac***: a classical illustration of response followed by tumor progression during continuing undiminished treatment with the same drug or combination of drugs. A nadir will be reached when the surviving tumor stem cell burden is composed of about 50% T/R cells (T/R to T/0 ratio = ca 1.0). Variations in the initial T/R to T/0 cell ratio in individual tumors will influence the degree and duration of response. ***Curve ad***: this curve would almost never be expected when treating patients with, for example, $\geq 10^9$ tumor stem cells with single drugs, because of the high probability of the presence of T/R cells with specific resistance. Burkitt's lymphoma and choriocarcinoma may be exceptions to this generalization. ***Curve e***: cure of relatively small Nos. of metastatic tumor stem cells after local surgery or radiation that left no T/R cells resistant to the drug employed.

models led to demonstrating the antibacterial action and mechanism of action of sulfonamides; PABA will prevent the antibacterial action of sulfonamides. This discovery eventually helped lead to the isolation and proof of the structure of folic acid and leucovorin and later to synthesis of aminopterin, methotrexate, and other antifols that are effective in treating certain experimental and human cancers. Furthermore, in vitro bacterial models in the hands of Hitchings and Elion played a major role in guiding the synthesis of purine antagonists that are effective in treating murine and human leukemias, particularly if drugs like 6-mercaptopurine or 6-thioguanine were administered in combination with non-cross-resistant drugs. In vivo experimental neoplasms were used in the discovery of 5-FU, floxuridine, cytarabine, hydroxyurea, and a variety of diverse alkylating agents, DNA binders, and mitotic inhibitors.

The astute deductions of Luria and Delbruck (1943) regarding the random mutation of bacteria from a state of virus sensitivity to virus resistance were (and are) of broad biological importance. Their experimental work was carried out in vitro with phage-infected *Escherichia coli* and their quantitative mathematical analyses led to the somatic mutation theory.

In the early 1950s, Burchenal and Law first selected specifically and permanently drug-resistant sublines of murine leukemia cells by repetitive passage in treated animals. Shortly thereafter, Law carried out his classical fluctuation test using the L1210 leukemia system in vivo. These experiments pointed to the origin of methotrexate-resistant leukemia cells and to the variations in the proportions of methotrexate-resistant cells in leukemia cell populations of the same size. Subsequent analyses indicated that the implications of the mutation theory apply in many experimental neoplasms in vivo. Thus, wide variations in the degree and duration of tumor response in similarly staged and treated animals or humans should not be surprising; they should be expected when failure is

due to survival and overgrowth of T/R cells.

In my opinion, a very important lesson learned in the clinic is just beginning to feed back to experimentalists and clinicians alike. It is the sad lesson learned in the depressingly slow stepwise progress in improving the designs of combination chemotherapy regimens to be used alone and in an adjuvant setting. It appears to me that we now must try to apply the lessons learned in formulating the mutation theory (and conceptually illustrated in the model of Goldie and Coldman) in the design of new combination chemotherapy regimens to be used in treating cancers comprised of varying mixes of T/0 and T/R cells. In this area of experimental therapeutics, bridging the gap between the laboratory and the clinic already has taken far too long (41+ years). I hope for some acceleration in bridging this gap before the year 2000; wise use of laboratory models almost surely would help and reduce our dependence on intuition.

### The Middle Years (1955–1975)

These were years of progress in cancer chemotherapy on almost all fronts—theoretical and very practical. Many new drugs were discovered and introduced. Significant progress was made toward the primary goal of research on cancer treatment, cure of patients bearing some disseminated cancers. Even so, a surprising number of individuals did not seem to realize it or did not choose to acknowledge it.

During the "middle years," biochemists gained information showing that the action of most anticancer drugs was directed toward biochemical events associated with cell division, eg, DNA replication and function or mitosis. It slowly became apparent that the response of experimental and human cancers to the available drugs was not due to some *qualitative* biochemical difference(s) between neoplastic and normal stem cells (as is the case between bacterial cells and mammalian stem cells in the sulfonamide model). Instead, it appears that the proliferative behavior of neoplastic and normal stem cells influences their relative sensitivity to cytotoxic drugs. (Bruce, Schabel, and their associates provided convincing data gained in experimental models.) This view also is supported by much data gained in animals and humans showing that the sites of limiting toxicity of most anticancer drugs are normal cell renewal systems (eg, marrow and intestinal epithelium). In addition, it is now quite clear that the presence of specifically and permanently drug-resistant tumor stem cells (T/R cells) is a major cause of chemotherapeutic failure. No one has ever seen evidence of the emergence and overgrowth of drug-resistant marrow or intestinal stem cells.

Important knowledge regarding the mechanisms of resistance of T/R cells to the agents that selected them was obtained during the middle years, along with much information regarding cross-resistance and lack of cross-resistance between different classes of drugs. Synthesis aimed at developing new non-cross-resistant drugs and models that will recognize them will be important in the years ahead.

Some investigators say that quantitative observations made with respect to the criteria of curability of experimental leukemias and other neoplasms by chemotherapy were helpful during the middle years (work of Schabel, Wilcox, and the author of this report, and Goldin, Venditti, and their associates). Zubrod, Frei, Freireich, DeVita, Holland, and other medical oncologists on the Acute Leukemia Task Force at the National Cancer Institute and members of Acute Leukemia Group B worked closely with experimentalists feeding lessons learned back and forth between the laboratory and the clinic. This was an unusually productive collaboration, possibly because experimentalists and clinicians regularly met and examined each other's data, concepts, and approaches in detail. The major lessons learned at the experimental level were: (*a*) there is an inverse relationship between the neoplastic cell burden and curability by any drug or combination of drugs, albeit with widely different total burdens curable in different neoplasms with different treatments; (*b*) dose response (or dose intensity response) is an invariable observation in experimental cancer chemotherapy; and (*c*) chemotherapeutic regimens must be designed so as to kill both T/0 and T/R cells faster than they regrow in intervals between treatment until all neoplastic stem cells are eradicated. Sometimes these concepts have been referred to collectively as the "cell kill theory" or the "first-order hypothesis."

### Recent Years, the Present, and the Future (1975–2000?)

By 1980, the inverse relationship between neoplastic cell burden and curability by chemotherapy had been clearly established in experimental cancers. This concept no longer was theory. Furthermore, large bodies of data suggested that the major reason for this inverse relationship was a direct relationship between neoplastic cell burden and the probability of the presence of limiting T/R cells—in keeping with the mutation theory and the model of Goldie and Coldman. Both of these concepts are compatible with the results of many internally controlled trials in which animals bearing different disseminated cancers were treated with surgery alone, chemotherapy alone, or surgery plus chemotherapy. Surgery plus chemotherapy is consistently superior to either treatment modality alone—if the metastatic burden is not too large (ie, does not already contain excessive numbers of limiting T/R cells that can survive the *particular* chemotherapy used).

By 1984, there could be little doubt of the superiority of combination chemotherapy (if delivered in a reasonable manner) over single-drug treatment. This holds for essentially all experimental neoplasms studied and many different human cancers. Almost surely the reason for

this is that non-cross-resistant drugs (if optimally delivered) will eradicate or help to hold limiting T/R cells in check. Having said this, let me pose a couple of questions and answer them in terms of lessons learned in experimental tumor systems.

1. Is it wise to sacrifice the dose intensity of the best available drugs against a neoplasm in order to increase the number of agents simultaneously administered?

In my opinion, the answer to this question often is "no." Again and again, experimental data show optimally delivered two- or three-drug combinations to be far superior to four-, five-, and six-drug combinations in which the dose intensity of the best two or three drugs was sacrificed in order to add more drugs to the combination.

2. What is the best way to deliver combination chemotherapy: simultaneously, in an alternating manner, or sequentially?

After analyzing the end results of many combination chemotherapy trials in different animal tumor systems, I have come to believe that this is not a very good question because it ignores some important variables that should be considered in designing any combination chemotherapy regimen. The lessons that I think have been learned have led me to this conclusion: in order for a combination of drugs to be maximally effective, it is critical that the *doses of the individual drugs be selected and matched* with respect to (*a*) the schedule to be used, (*b*) the growth rate of the neoplasm, and (*c*) *the expected proportions of limiting T/R cells*.

In experimental neoplasms, the proportions of T/R cells with resistance to specific antimetabolites are consistently higher than the proportions with resistance to specific alkylating agents. Thus, if a simultaneous combination contains an antimetabolite and an alkylating agent, it might be wise, if necessary, to sacrifice dose intensity of the former.

Before terminating this mini-review on laboratory models and lessons learned from them, and on translating from the clinic to the laboratory and vice versa, I might as well be candid. (I am old enough to be allowed this privilege—at least occasionally.)

Choosing laboratory models to be used in a routine manner for "screening" is a difficult task because, before the fact, one cannot know what good or poor agents will be selected or missed by any model as it is set to operate. Choosing a screening model (or models) that would satisfy everyone with an opinion is well-nigh an impossibility. In my opinion, how one uses a model and interprets the results obtained with it often is as important as choosing the model. If you set the criteria for "activity" too low for any screening system, you surely will select some clinical false-positives. If you set the criteria much higher, you will miss some agents that might be effective against some human cancer(s).

I feel fairly confident about lessons learned regarding experimental therapeutics in various experimental tumor systems and in the clinic—and translation in both directions. The experimental and clinical data I have studied and attempted to analyze over the past 10 years have led me to these working concepts:

1. The presence of diverse T/R cells, or their emergence during treatment, is a major cause of chemotherapeutic failure (ie, single-drug treatment and combination chemotherapy used alone or in an adjuvant setting).

2. In classification of different cancers by chemotherapeutic effect (sensitive, refractory, or very refractory), a major underlying difference is in the proportions of a wide spectrum of limiting T/R cells at a given tumor size.

3. A major reason for variations in the degree and duration of response of a given neoplasm, in similarly staged and treated individuals, is fluctuation in the proportions of limiting T/R cells.

Today some investigators are wondering if we are in a temporary or permanent plateau with respect to progress in cancer chemotherapy—at least with the drugs now at hand. By this, I assume that they mean that the prospects of curing more patients with additional types of disseminated cancers, using combination chemotherapy in conjunction with surgery or radiation, are poor.

Before I could accept this view, I would first have to be convinced that the presence of T/R cells, or their emergence during treatment, is *not* a major cause of chemotherapeutic failure when used alone or in an adjuvant setting. Furthermore, I would like to feel sure that we have done all that can be done with optimally designed combination chemotherapy regimens used in conjunction with local treatment. This might require reexamination of dozens of combination chemotherapy protocols that already have been used in treating disseminated human cancers and planning some new ones, *bearing in mind the importance of the dose intensity of each drug in a combination* with respect to the potential of eradication of the T/0 cells *and* the T/R cells that are specifically resistant to each drug in the combination.

Have in vivo experimental models provided any lessons that might be useful in the design of future combination chemotherapy regimens? In answer to this question, I will repeat what analyses of many experimental combination chemotherapy trials indicate to me. It is critical to select and match *the dose intensity of each drug* in a combination with attention to (*a*) the schedule to be used, (*b*) the growth rate of the neoplasm, and (*c*) the expected proportions of limiting T/R cells.

# New Approaches to Antitumor Drug Screening: The Human Tumor Colony-Forming Assay[1]

**Robert H. Shoemaker**[2,3,*]

Historically, antitumor drug discovery and development efforts have relied on in vivo tumor models. At the National Cancer Institute (NCI), two murine leukemia models, L1210 and P388, have constituted the primary screens in which more than 350,000 unique chemical compounds have been tested over a period of 30 years (1,2). Additional murine tumors, and more recently some human tumor xenograft models, have been used to supplement these primary models. While this program has been successful in identifying clinically active anticancer drugs and has, in fact, been either directly responsible for, or played a major role in, the discovery and development of the majority of drugs currently approved for clinical use (3), the drugs identified have shown rather limited value in treatment of the common solid tumors such as lung, colon, breast, or prostate cancer. The initial reports of Hamburger and Salmon (4) and Salmon et al (5) describing the selective cloning of human tumors in primary culture for drug sensitivity testing (the human tumor stem cell assay) generated a great deal of interest and stimulated many investigators to explore the basic biology of the assay as well as its application in predicting the response of individual patients to chemotherapy. Several review articles on this subject have been published recently (6-9). While some controversy regarding the validity of the assay continues (7,10), results of retrospective clinical correlations have shown reasonable predictive accuracy, especially for identifying drug-resistant tumors (11-13). Relatively limited data are as yet available from randomized prospective trials with the assay. In some instances, these studies have yielded rather disappointing results, especially in relation to in vitro cloning performance (14), and in others, a positive therapeutic advantage seems to be associated with treatment based on the in vitro assay results (15). In considering these results, it should be noted that several other in vitro assay approaches have been utilized which yield similar predictive accuracy (16). The assay described by Weisenthal et al (17,18) stands out as one with particular promise, including an indication of value in prospective trials of patients with leukemia (19).

The potential value of the human tumor stem cell assay in new drug development was suggested by Salmon (20) shortly after the initial reports on the assay. The first such applications of the assay by his and other groups involved analog comparisons (21,22) and "in vitro phase II trials" (23,24). The NCI has organized a contract-based, multi-institutional cooperative program to evaluate the assay for de novo drug screening and as a tool for new drug development. The organization of this program and preliminary results have been published previously (25,26). In this paper, I will present an overview of the NCI experience, describe two novel compounds identified in screening with the assay, and discuss possible future uses for the assay.

## A Reliable Protocol for New Drug Screening

The human tumor stem cell assay, as originally described (4,5), is subject to a variety of artifacts. These have been noted in review articles (7,9) and discussed in considerable detail (10,27). Preparation of single-cell suspensions from solid tumor specimens is a technically difficult process. In practice, each tumor specimen requires individualized attention for optimal processing. Even under the best of conditions, eg, highly cellular viable specimens with minimal stroma, true single-cell suspensions are rarely achieved. Because of this, variable numbers of cell aggregates are included in the initial cultures. In recognition of this, the term human tumor colony-forming assay (HTCFA) seems the most appropriate name for the assay.

To ensure that each assay yields meaningful results, it is essential that quality control measures are built

[1]Drug screening was performed under contracts N01-CM-07251, N01-CM-07327, N01-CM-07419, and N01-CM-07420 (with the University of California at Los Angeles, the Mayo Foundation, the University of Arizona, and the Cancer Research and Therapy Foundation of South Texas) from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

[2]Drug Evaluation Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[3]The protocol for new drug screening with the human tumor colony-forming assay cited in this paper was developed in collaboration with Drs. David Kern, Michael Lieber, Sydney Salmon, and Daniel Von Hoff.

[*]*Reprint requests to:* Dr. Robert H. Shoemaker, NCI-Frederick Cancer Research Facility, Bldg 428, Rm 63, Frederick, MD 21701.

into the HTCFA protocol. These measures should establish that a reasonable cell suspension is used to initiate the assay, that real cellular proliferation occurs over the course of the incubation period, that control growth is measured with reasonable accuracy, and that a lethal treatment will result in significant measured inhibition of colony formation. A set of quality control parameters which meet these requirements has been described previously (25,27). In order to maximize assay sensitivity, the NCI screening project has utilized "continuous" drug exposure. This has allowed considerable simplification of the assay procedure, ie, drug solutions can be applied to the surfaces of replicate soft agarose plates rather than to multiple tubes containing cell suspensions which are subsequently washed, then combined with agarose and added to culture plates. The advantages of this technique have been described in detail (28).

An in situ vital staining procedure (29,30) has been built into the NCI protocol for the HTCFA. This not only provides a more appealing endpoint, ie, viable colony count, but increases assay sensitivity and improves the performance of the automated image analysis system (31) used for counting colonies. Evaluation of viable colony number is an effective approach to minimizing the artifactual influence of input aggregates on the assay.

## Results of New Drug Screening With the HTCFA

A key question regarding the use of the HTCFA for drug screening is whether it will identify promising new antitumor drug leads which would be missed by the traditional in vivo screening method. This has been approached by selecting compounds for HTCFA screening which had been previously tested and found to be negative in the pre-screen currently used by NCI (P388 in vivo). Preliminary results of this testing have been reported (25,26). While a rather small number of these compounds have been tested to date, it is clear that the HTCFA is effective in identifying cytotoxic compounds which are not active in the in vivo P388 prescreen. The basis for the lack of in vivo P388 activity has been explored for a subset of the HTCFA active compounds. In some instances, a relatively trivial explanation was identified, eg, the formulation used for the in vivo testing was suboptimal (for insoluble compounds, a hydroxypropyl-cellulose suspension is used). In other cases, evidence for rapid metabolic inactivation of the compound was obtained from in vitro hepatocyte co-culture studies (unpublished observations). The compounds currently considered of greatest interest are those which are relatively noncytotoxic to P388 cells in vitro (indicating that the lack of in vivo activity may be explained by lack of target cell sensitivity), show in vivo activity in some other tumor model, or demonstrate significant evidence for selective in vitro cytotoxicity for particular tumor types. Of approximately 300 prescreen negative compounds which have entered the HTCFA screening program, eight (approximately 3%) meet at least one of the above criteria.

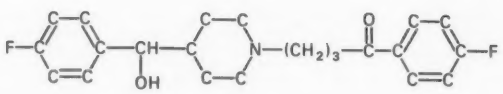

FIGURE 1.—Structure of dihydrolenperone.

## Antitumor Drug Leads From HTCFA Screening

As mentioned above, eight compounds from HTCFA screening are currently considered as interesting antitumor drug leads. Two of these compounds have been studied in considerable detail and have been accepted into the NCI's formal drug development program.

The structure of dihydrolenperone is shown in figure 1. This compound shows some evidence for in vitro selective cytotoxicity in lung cancer (table 1) and has shown in vivo activity in the L1210 leukemia model. While the level of L1210 activity observed is modest (maximum increase in lifespan 37%), this activity is retained when the compound is administered by the oral route. Remarkably, this compound, which was acquired by NCI through routine industry liaison, was found to have been taken to clinical trial previously by the supplier (Merrell-Dow) as an antipsychotic drug. Merrell-Dow lost interest in the compound because of side effects (hypotension) and the lack of outstanding antipsychotic activity. Dihydrolenperone is planned for clinical trials, as an oral formulation, beginning in lung cancer.

Chloroquinoxaline sulfonamide, shown in figure 2, is the first sulfanilamide derivative to be seriously considered as an antitumor drug. This compound is not inactivated in hepatocyte co-culture, shows greater activity against human tumors in vitro than against P388 cells in vitro, and shows therapeutic activity in three in vivo models, including two new human tumor xenograft models which utilize survival as endpoint (32,33). Toxicology studies are planned for this compound in the near future.

## Future Directions for In Vitro Screening

Experience in screening with the HTCFA has shown that interesting antitumor drug leads are identified

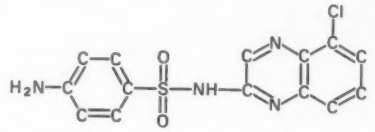

FIGURE 2.—Structure of chloroquinoxaline sulfonamide.

TABLE 1.—HTCFA screening data for dihydrolenperone

| Concentration (μg/ml) | In vitro response rate by tumor type* | | | | | |
|---|---|---|---|---|---|---|
| | Breast | Colorectal | Kidney | Lung | Melanoma | Ovary |
| 10 | 0/6 | 1/11 | 0/11 | 7/17 | 1/14 | 2/18 |
| 1 | 0/6 | 0/11 | 0/10 | 2/17 | 0/11 | 0/11 |
| 0.1 | 0/6 | 0/11 | 0/10 | 3/15 | 0/9 | 0/10 |

*An in vitro "response" is defined as reduction of colony formation to ≤ 30% of the control value at the indicated test concentration.

which would not be detected by the traditional in vivo screening approach. The quality of these drug leads, of course, will not be known until clinical trials have been performed. Experience with the HTCFA has also shown that it is not feasible for large-scale screening and that with current technology its use is confined to a limited number of tumor types (25). Further improvements in assay technology, such as the use of a radioisotope endpoint (34,35) or perhaps the capillary tube cloning method (36,37), may enhance the utility of the assay, but it does not seem likely that these will permit truly large-scale screening with the assay.

NCI is currently working on development of a new in vitro screening model based on the use of established tumor cell lines. This model has been conceived as a "disease-oriented" one in which panels of tumor cell lines will be used to represent disease (tumor) types. The initial focus of this effort is on lung cancer, employing cell lines developed by Gazdar et al (38) and Carney et al (39). Automated growth inhibition assays (40,41) compatible with very large-scale screening are being evaluated for use in this model. If successful, this approach will be extended to include modeling of other tumor types (colon, breast, and prostate cancer). Once this model is developed, it will be possible to make a comparison of its screening performance with that of the HTCFA. Clearly, what is sought in both models are compounds which exert selective cytotoxicity for particular tumor types. Confirmation of selectivity indicated by the cell line panel using fresh tumor material from a significant number of different patients may be the most appropriate future role for the HTCFA.

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# Relationship of Structure to Anticancer Activity and Toxicity of the Nitrosoureas in Animal Systems

**Thomas P. Johnston and John A. Montgomery[1,*]**

An understanding of the chemistry of the nitrosoureas is essential to an understanding of their biologic activity, although their chemistry alone certainly does not explain their apparent specificity for neoplastic cells and the resulting anticancer activity they display against both experimental animal neoplasms and human cancers.

Other similar *N*-nitroso compounds have shown no anticancer activity (table 1) even though some are quite toxic, mutagenic, or carcinogenic and therefore biologically active (1). More recently, however, the curative action of certain 2-chloroethyl nitrosourethanes in the L1210 leukemia system has been demonstrated (2) and *N*-(2-chloroethyl)-*N*-nitroso-*N'*-nitroguanidine has considerable activity in this system, albeit less than the corresponding urea (3). At the same time, none of the precursors of these *N*-nitroso compounds, including the ureas, are very active biologically. This clearly indicates the necessity for the *N*-nitroso group, which labilizes the bond between the nitrosated nitrogen and the adjacent atom, so that when the adjacent atom is a carbon double-bonded to a heteroatom, decomposition occurs spontaneously in aqueous media, the rate depending on the particular structure and the pH of the medium (4,5). Breakage of this bond, regardless of the exact mechanism, gives rise to a diazotate (fig 1) capable of alkylating molecules such as DNA under physiologic conditions of pH, ionic strength, and temperature. In the case of the *N*-nitrosoureas the reaction may be initiated by loss of a proton from the unnitrosated nitrogen. In support of this mechanism is the stability of the trisubstituted *N*-nitrosoureas, which are not toxic to cells in culture and must be metabolized, dealkylating in the liver, to show in vivo activity (6–8). In the case of *N*-methyl-*N*-nitrosourea, the first nitrosourea found to have anticancer activity, this decomposition leads to methyldiazotate and isocyanic acid (4), compounds capable of methylating and carbamoylating biological macromolecules. It is now known that other *N*-nitroso compounds such as *N*-nitrosoamides and *N*-nitrosourethanes that produce alkylating moieties but not carbamoylating moieties have anticancer activity, and at the same time, certain nitrosoureas that have little carbamoylating activity are quite active (1), whereas nitrosoureas with no detectable alkylating activity are very weakly active or inactive (table 2). Thus, alkylating activity, but not carbamoylating activity, appears to be essential, although there is not a straight line correlation between alkylating and anticancer activity (9,10). Carbamoylation has been associated with the inhibition by the nitrosoureas of the repair of DNA damaged by ionizing radiation or by alkylating agents (11,12) and thus could indirectly contribute to the cytotoxicity and anticancer activity of these compounds.

The kinetics of cell killing by the nitrosoureas, like that of other alkylating agents, is pseudo first order since the drug is always present in large excess (fig 2A). There is a small difference in the responsiveness of resting and dividing cells, with dividing cells being two or three times as sensitive (fig 2B). Cells are essentially equisensitive in all phases of the cell cycle and cell killing is rapid. This behavior is in contrast to that of the antimetabolites such as cytarabine (ara-C), the cell killing by which is, above a certain minimum concentration, concentration-dependent (fig 3). Ara-C is an S-phase specific agent requiring long exposure relative to carmustine (BCNU) for maximum cell kill. These differences are the basis for the schedule dependency of these agents (13) in the treatment of mice inoculated with L1210 leukemia (table 3). The nitrosoureas are most effective when given as a single dose, whereas ara-C must be given every 3 hours to realize its potential. Other antimetabolites, such as methotrexate, 6-mercaptopurine, and 5-FU, are maximally effective on a daily or every other day schedule (13).

## Anticancer Activity

### *General Considerations*

The data in table 2 show that the most active nitrosoureas in the L1210 system contain either the *N*-methyl-*N*-nitrosoureido or the *N*-(2-haloethyl)-*N*-nitrosoureido function. Table 4 compares these two structural types: the methyl versus the 2-chloro- or 2-fluoro-ethyl. The 2-haloethyl compounds are from two to 20 times as toxic as the methyl compounds, but are much more active against

[1]Southern Research Institute, Birmingham, AL.
* *Reprint requests to:* Dr. John A. Montgomery, Southern Research Institute, 2000 9th Ave, PO Box 55305, Birmingham, AL 35255-5305.

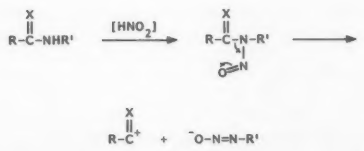


FIGURE 1.—Structure of a diazotate.

L1210 leukemia, giving 50%-100% cures with a single injection. The methyl compounds are more stable and have only one-tenth to 1/10 as much alkylating activity. The carbamoylating activity is more dependent on the nature of R than R'. These data also support the concept that alkylation is the most important factor to activity, with the 2-haloalkyl compounds being the best alkylating agents. The problem is more complicated than that, however, because the chemistry of the 2-haloalkyl compounds is more complex (fig 4) due to the lability of the carbon-halogen bond (Br > Cl > F). Kohn (14) and Ewig and Kohn (15) have proposed that a two-step crosslinking of DNA (fig 5) is important to the high level of activity of these compounds, even though the amount and rate of crosslinking by the 2-chloroethyl compounds are much greater than by the 2-fluoroethyl compounds due to their greater chemical reactivity. There is evidence that the initial attack of (2-haloethyl)diazotate occurs at $O^6$ of a guanine residue of DNA followed by cyclization to $N^1$ with loss of halogen. Attack by $N^3$ of an adjacent cytosine residue on this cyclic intermediate then gives the DNA crosslink [1-(*N*$^3$-deoxycytidyl)-2-(*N'*-deoxyguanosinyl)-ethane] (16,17). All of this information led to the postulation, which has now been established (2,18,19), that other types of 2-haloethylating agents with a high level of activity in experimental animal tumor systems could be developed. The value of these new compounds in the treatment of human cancer has yet to be established.

From a comprehensive study of 96 2-haloethyl nitrosoureas, Hansch et al (20) derived equations relating activity, as judged by a 3-log kill of L1210 cells in vivo, and toxicity (LD10) to log P. Although the *r* and *s* values are reasonably good for a study of this magnitude, no confidence limits could be placed on log $P_0$ for either activity or toxicity and, thus, there is no clear separation of toxicity from activity as was hoped. The most active compounds are also the most toxic compounds, but at the same time they have the greatest therapeutic index. Furthermore, there is a log $P_0$ range for maximum activity even though it is fairly broad. Data from approximately 300 compounds prepared at the Southern Research Institute show that the ED50 (the dose required to cure 50%

**Table 1. Activity of Various N-Nitroso Compounds vs. Leukemia L1210 ($10^5$ cells, i.p.)**

| RN(NO)R' | | Dose | Treatment | |
|---|---|---|---|---|
| R | R' | mg/kg/dose | Schedule | % ILS[a] |
| $O_2NHNC(=NH)-$ | Me | 16 | Chronic | 30 |
| $H_2NCO-$ | Me | 12 | Chronic | 109 |
| MeHNCS– | Me | | Chronic | Inactive |
| $EtO_2C-$ | Me | | Chronic | Inactive[b] |
| Me | Me | | Chronic | Inactive[c] |
| $MeSO_2$ | Me | | Chronic | Inactive |
| R"[d] | $SO_2Me$ | | Chronic | Inactive |
| $O_2NHNC(=NH)-$ | $(CH_2)_2Cl$ | 20 | Chronic | 189 |
| R"HNCO– | $(CH_2)_2Cl$ | 4-750 | Day 1 | 10/10[e] |
| $EtO_2C-$ | $(CH_2)_2Cl$ | | Chronic | Inactive[f] |
| R"CO– | $(CH_2)_2Cl$ | 18-180 | Chronic | 1 to 65 |
| $MeC_6H_4SO_2HNCO-$ | $(CH_2)_2Cl$ | 90 | Day 1 | 4/6[e] |
| $MeC_6H_4SO_2-$ | $(CH_2)_2Cl$ | | Chronic | Inactive |

[a]Increase in lifespan.

[b]Toxic.

[c]Carcinogenic.

[d]R" = various alkyl or cycloalkyl groups.

[e]Cures/total number of animals.

[f]Highly toxic.

of a treated group of mice inoculated with L1210 leukemia; $10^5$ cells ip) varies 100-fold (from 0.024 to 2.3 mmols/kg), whereas the therapeutic index, defined as the LD10/ED50, varies less than fourfold (from 1 to 3.9 mmols/kg), again supporting the idea that the most potent compounds are also the most toxic. In fact, the compounds with a high LD10 (2.4–2.7 mmols/kg) are at the low end of the therapeutic index range.

### *Latentiated Nitrosoureas*

The clinical nitrosoureas are characterized by a 2-chloroethyl group on the nitrosated nitrogen and monosubstitution on the other nitrogen by groups that can impart distinctive chemical properties. It is the abstraction of the remaining proton under physiological conditions which initiates the easy decomposition into alkylating and carbamoylating species that accounts for the biological effects observed (21–23). Such decompositions are blocked by full substitution, which usually results in stabilization but not always in inactivity. An explanation for the biological activity of fully substituted nitrosoureas is provided by the concept of latentiation, which is the in vivo release of a biologically active compound from a suitably derivatized form (24). Full substitution as in the piperazinedicarboxamide (fig 6, No. *1*), however, resulted in inactivity against murine L1210 leukemia (25) and stability in aqueous solution under physiological conditions (26). These results discouraged like synthesis until carcinogenicity in rats was observed (27,28) with *N*-ethyl-*N'*, *N'*-dimethyl-N-nitrosourea (fig 6, No. *2*) and *N*-butyl-*N'*, *N'*-dimethyl-N-nitrosourea (fig 6, No. *3*) and other trialkylnitrosoureas, presumably as a result of enzymatic activation.

Latentiation of actual and potential antineoplastic nitrosoureas was effected when one of the substituents was a suitably positioned methyl group, as in *N*-(2-chloroethyl)-*N'*-methyl-*N*-nitroso-*N'*-propylurea (fig 7, No. *4*),

**Table 2. The Relationship of Activity to Structure and Chemical Properties of Nitrosoureas**

| RNHCON(NO)R' | | | | | Leukemia L1210[d] | |
|---|---|---|---|---|---|---|
| R | R' | $T_{\frac{1}{2}}$, a min. | Alkylating Activity, $A_{540}$[b] | Carbamoylating Activity, dpm[c] | $LD_{10}$ or OD[e] (mg/kg) | % ILS[f] |
| H | Me | 7.0 | 0.36 | 10,890 | 12[g] | 109 |
| $C_6H_{11}$ | Me | 486 | 0.016 | 19,800 | 300 | 27 |
| $(CH_2)_2Cl$ | Me | 254 | 0.020 | 8,850 | 150[g] | 46 |
| glucos-2-yl | Et | 46 | ca. 0 | low | --- | I[h] |
| Et | Et | 202 | 0 | 13,730 | 400 | 44 |
| $NC(CH_2)_2$ | $(CH_2)_2CN$ | 32 | 0.04 | 7,000 | 150 | 55 |
| $C_6H_{11}$ | $C_6H_{11}$ | 5.6 | 0 | 36,540 | --- | I |
| $ClCH_2(Et)CH$ | $CH(Et)CH_2Cl$ | 15 | 0.3 | 25,140 | --- | I |
| $ClCH(Me)CH_2$ | $CH_2(Me)CHCl$ | 41 | 0.04 | 10,290 | 1000 | 140 |
| $C_6H_{11}$ | $CH_2(Me)CHCl$ | 53 | 0.013 | 26,270 | 750 | 87 |
| R | $(CH_2)_2F$ | 9 - 68 | 0.6 to >3 | 0 - 49,000 | 6 - 89 | Curative |
| R | $(CH_2)_2Cl$ | 1.3 - 61 | 0.3 to >3 | 800 - 42,000 | 4 - 600 | Curative |
| R | $(CH_2)_2Br$ | --- | --- | --- | 200 - 300 | Curative |
| R | $(CH_2)_2I$ | --- | --- | --- | --- | I |

[a] Half-life in ethanol/phosphate buffer (1/19). pH 7.4, 37°.

[b] $A_{540}$ is a measure of the concentration of alkylated 4-(4-nitrobenzyl)pyridine in an ethyl acetate extract of a mixture of the nitrosourea and the pyridine in acetate buffer, pH 6.0, that had been incubated at 37° for 2 hr.

[c] The dpm is a measure of radioactivity present in unidentified reaction products obtained upon incubating the nitrosourea with $^{14}C$-lysine in phosphate buffer, pH 7.4, at 37° for 6 hr.

[d] $10^5$ cells implanted i.p.

[e] Lethal $Dose_{10}$ or Optimal Dose.

[f] % Increase in lifespan.

[g] Chronic treatment.

[h] Inactive.

which was shown to be relatively stable in aqueous solution and active against L1210 leukemia only in vivo, an observation indicative of enzymatic demethylation (6–8). Compound *4* (fig 7, No. 4) had the highest therapeutic index of a series that included methyl, 2-chloroethyl, and cyclohexyl groups as R′. The morpholine derivative *5* (fig 8, No. *5*) was similarly active against L1210 leukemia in mice (29) and L5222 leukemia in rats, effecting some cures and being described as remarkably nontoxic (30). The dimethylmorpholine derivative *6* (fig 8, No. *6*) in the latter study was distinctly less active than *5*. 2-Chloroethyl groups in such trialkylnitrosoureas, however, enhanced toxicity in rats, tris(2-chloroethyl)nitrosourea (fig 7, No. *7*) being particularly toxic and also carcinogenic (31).

In a study of (2-chloroethyl)nitrosoureas derived from amino acid amides as carriers (32), the high activity of the sarcosinamide derivative *8* (fig 7, No. *8*) against L1210 leukemia at a high dose could be the result of enzymatic demethylation; even though decomposition of *8* in phosphate buffer afforded sarcosinamide as the sole ninhydrin-positive product, such decomposition was slow (half-life, 330 minutes). The prolinamide derivative *9* (fig 8, No. *9*) was almost as stable, but less antileukemic and

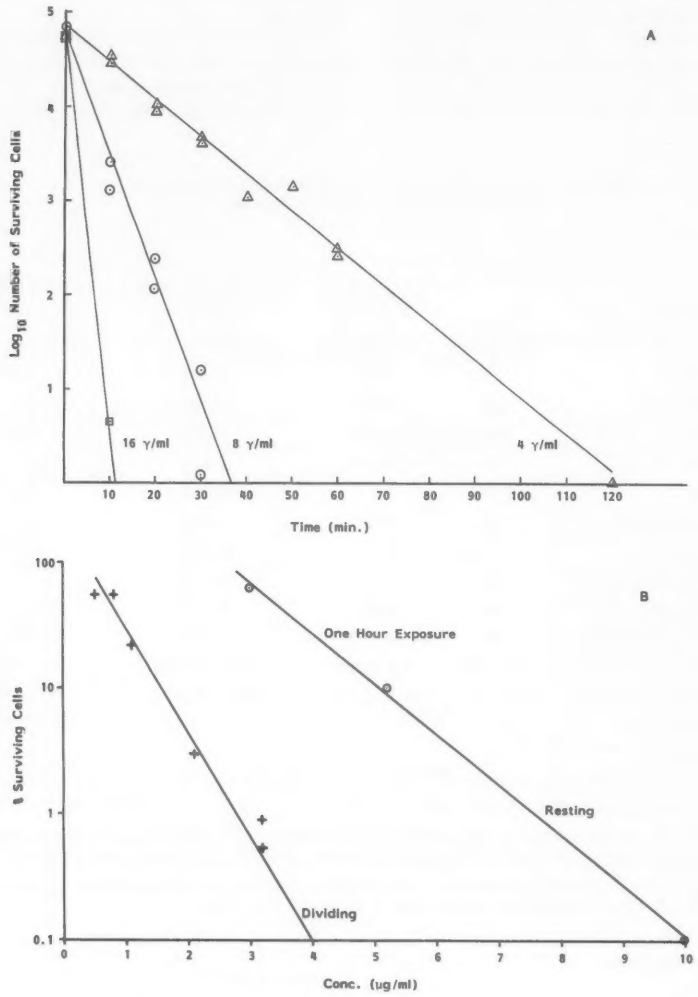



FIGURE 2.—Effect of BCNU on L1210 cells in culture.

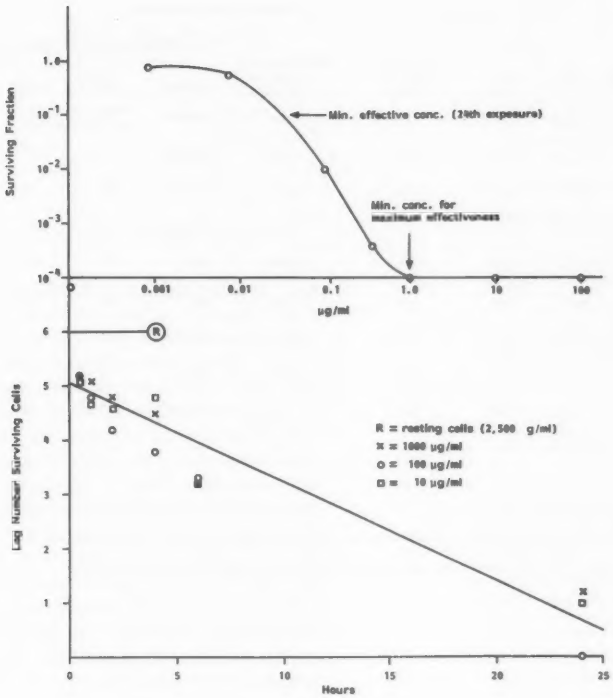



FIGURE 3.—Effect of ara-C on L1210 cells in culture.

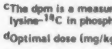
**Table 3. Schedule Dependency of Several Anticancer Agents in the L1210 Leukemia System**

| | % ILS (Cures/Treated)[a] | | | | |
|---|---|---|---|---|---|
| | Single Dose, Day 2 | q3h x 8, Days 2,6, 10, and 14 | qd 2-16 | q2d, 2-16 Days | q4d, Days 2,6, 10, and 14 |
| BCNU | >100 (40/50)[b] | >100 (12/30) | 50 (1/20) | >100 (5/10) | >100 (6/10) |
| CCNU | >100 (357/425)[b] | >100 (6/60) | 33 (0/10) | >100 (5/10) | >100 (18/40) |
| Cyclophosphamide | >100 (44/79)[b] | 20 (0/10) | 38 (0/30) | 60 (0/10) | >100 (0/10) |
| Daunomycin | 33 (0/10) | 25 (0/30) | 27 (0/10) | 16 (0/10) | 33 (0/10) |
| Methotrexate | 50 (0/30) | 69 (0/30) | >100 (0/120)[b] | >100 (0/30)[b] | >100 (0/80)[b] |
| 6-Mercaptopurine | 39 (0/20) | 46 (0/20) | 60 (0/80)[b] | 33 (0/10) | 44 (0/10) |
| 5-Fluorouracil | 50 (0/20) | 100 (0/30) | >100 (0/20)[b] | >100 (0/20)[b] | 83 (0/10) |
| Ara-C | 59 (0/20) | >100 (135/155)[b] | >100 (0/100) | >100 (0/20) | >100 (0/10) |

[a]Median % increase in life span (excluding survivors) and cure rate for highest nontoxic dose for each schedule.

[b]Optimal schedule.

Table 4. Comparisons of N-Methyl- and N-(2-Haloethyl)-N-nitrosoureas

| RNHCONH(NO)R' | | | | | Leukemia L1210 ($10^5$ cells, i.p.) | | | |
|---|---|---|---|---|---|---|---|---|
| R | R' | $T_{\frac{1}{2}}$,[a] min. | Alkylating Activity $A_{540}$[b] | Carbamoylating Activity dpm[c] | Schedule | $LD_{10}$ or OD[d] | % ILS | Cures[e] |
| H | Me | 7.0 | 0.36 | 10,890 | Chronic | 12 | 109 | 0 |
| | | 1.3 | 3.0 | 8,560 | Chronic | 0.90 | 147 | 5-10[f] |
|  (X=F, Cl) | Me | 250 | 0.02 | 8,850 | Chronic | 150 | 46 | 0 |
| | | 43 | 1.4 | 28,700 | Chronic | 8.0 | 187 | 5-10[f] |
| | | N.D. | 1.3 | 19,800 | Day 1 | 12 | 233 | 8 |
|  | Me | 490 | 0.02 | 19,800 | Day 1 | 300 | 27 | 0 |
| | | 53 | 0.52 | 42,000 | Day 1 | 48 | --- | 10 |
|  |  | 68 | 0.64 | 49,100 | Day 1 | 34 | --- | 5-10 |
| | Me | 48 | 0.19 | 1,630 | qd 1-9[g] | 50 | 31 | |
| |  | 21 | 2.4 | 820 | Day 1 | 29 | --- | 9 |
| |  | | | | | | | |

[a]Half life in ethanol/phosphate buffer (1/19), pH 7.4, at 37°.

[b]$A_{540}$ is a measure of concentration of alkylated 4-(p-nitrobenzyl)pyridine in an ethyl acetate extract of a mixture of the nitrosourea and the pyridine in acetate buffer (pH 6) incubated at 37° for 2 hours.

[c]The dpm is a measure of radioactivity present in unidentified reaction products obtained upon incubation of the nitrosourea with lysine-$^{14}$C in phosphate buffer (pH 7.4) at 37° for 6 hours.

[d]Optimal dose (mg/kg/dose).

[e]Out of 10 treated animals.

[f]Cures from a single injection on day 1.

[g]Optimal schedule.

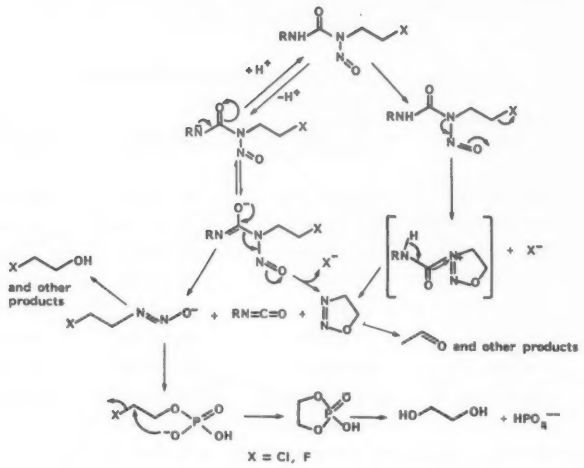



FIGURE 4.—Aqueous decomposition of the 2-haloethylnitrosoureas.

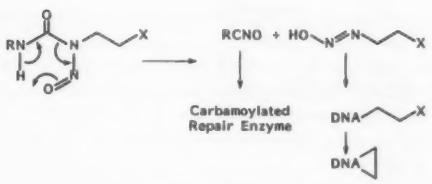
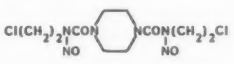



FIGURE 5.—Important reactions of the 2-haloethylnitrosoureas.

more toxic. Similar results were obtained with the sarcosinamide and prolinamide derivatives *10* and *11* (fig 7, No. *10*; fig 8, No. *11*) in a series in which the amide nitrogen was substituted with a 2-chloroethyl group (33), but the prospect of enhanced activity with such a modification was minimal in view of the reported inactivity of *N*-(2-chloroethyl)butanamide against L1210 leukemia (34).

It has also been demonstrated that latent activation can be effective against an experimental solid tumor: *cis*-4-[[[(2-chloroethyl)nitrosoamino]carbonyl]methylamino]-cyclohexanecarboxylic acid (fig 7, No. *12*) effected a significant cure rate of both early and established murine Lewis lung carcinoma even though its in vitro half-life was about five and one-half times that of the unmethylated parent compound (26). As with other enzymatically activated nitrosoureas, the increased stability and reduced toxicity enabled the use of the large doses required for maximal effectiveness, which have yet to be correlated with the rate and efficiency of dealkylation.

An expanded study of *N'*,*N'*-disubstituted analogs that also included *5* above led to the discovery that those with suitably positioned hydroxyl groups, as in *N*-(2-chloroethyl)-*N'*,*N'*-bis(2-hydroxyethyl)-*N*-nitrosourea (fig 7, No. *13*), and a number of similar compounds, were unstable in phosphate-buffered aqueous solution and highly active against L1210 leukemia without prior enzymatic activation (29). These properties were attributed to a chemical activation initiated by attack of a β-hydroxyl group on the ureido carbonyl group with *N*-substituted-oxazolidinone formation and thus without the coformation of a carbamoylating species. These compounds, called a new class of nitrosoureas, were also active against Ehrlich ascites murine carcinoma, whereas those requiring enzymatic activation were hardly active against this tumor.



1

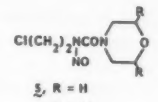
2, R = Et
3, R = Bu

FIGURE 6.

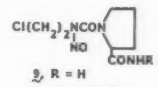
4, R = Me; R' = Pr
7, R = R' = $(CH_2)_2Cl$
8, R = Me; R' = $CH_2CONH_2$
10, R = Me; R' = $CH_2CONH(CH_2)_2Cl$
12, R = Me; R' =
13, R = R' = $(CH_2)_2OH$
14, R = $CH_2$ ; R' =
15, R = $(CH_2)_2OMe$; R' =
16, R = $(CH_2)_2OMe$; R' =
17, R = Me; R' =
18, R = Me; R' =
19, R = $CH_2CHOHCH_2OH$; R' =
20, R = $CH_2CHMe_2$; R' =

FIGURE 7.

The initial group of seven hydroxyalkyl-substituted nitrosoureas was immediately extended to a large number of glycopyranosyl derivatives (35,36) in which both *N'*-substituents were varied in a continued search for reduced myelotoxicity and strong antitumor activity among water-soluble nitrosoureas related to chlorozoto-



5, R = H
6, R = Me



9, R = H
11, R = $(CH_2)_2Cl$

FIGURE 8.

cin and GANU. The galacto- and arabino-pyranosyl derivatives in particular showed remarkable activity against L1210 leukemia in comparison with positive controls, outstanding examples being the galactopyranosyl derivative ***14*** (fig 7, No. ***14***) and the arabinopyranosyl derivative ***15*** (fig 7, No. ***15***). Similar high antitumor activity was seen in positional isomers (37,38) such as methyl 3-[[[(2-chloroethyl)nitrosoamino]carbonyl](2-methoxyethyl)amino]-3-deoxy-α-D-glucopyranoside (fig 7, No. ***16***) and the C-4 isomer ***17*** (fig 7, No. ***17***), but positional isomers (39) like the C-6 isomer ***18*** (fig 7, No. ***18***) were significantly less active than the C-2 and C-3 isomers. The rate of activation by a γ-hydroxyl group in C-6 isomers proved much slower than that by a β-hydroxyl group in C-1 or C-3 isomers. All congeners in a series of *N′*-substituted *N*-(2-chloroethyl)-*N′*-(*trans*-2-hydroxycyclohexyl)-*N*-nitrosoureas were strongly active against L1210 leukemia, but only those having two kinds of β-hydroxyl groups as in the *N′*-2,3-dihydroxypropyl derivative ***19*** (fig 7, No. ***19***) were equally as active against Ehrlich ascites carcinoma, suggesting differences in mode of activation (40).

Further modification of this type of nitrosoureas by the introduction of disaccharide groups led to the observation that a large number of the 30 maltosyl derivatives studied were curative (60-day survival) of L1210 leukemia, whereas lactosyl and melbiosyl examples were almost inactive (41). Furthermore, the most interesting compound in this series, the maltosyl derivative ***20*** (fig 7, No. ***20***), showed a significantly higher therapeutic ratio against L1210 leukemia when administered in five daily doses than when administered in a single dose, which appears to be optimal for standard nitrosoureas (42). Acetylation of ***20*** resulted in a substantial loss of activity, supporting the requirement of a free hydroxyl group for activation. The selective activity of ***20*** may be related to the fact that only maltose among disaccharides is known to be metabolized in common mammalian cells (43) and was shown (44) to be converted by maltase to *N*-(2-chloroethyl)-*N′*-(β-D-glucopyranosyl)-*N′*-(2-methylpropyl)-*N*-nitrosourea, a derivative of GANU. On the basis of the above studies and others (45), in which good activity against a spectrum of tumors including advanced Lewis lung carcinoma was demonstrated in comparison with GANU, ACNU, and lomustine (CCNU), ***20*** appears to be a unique nitrosourea and is under chemical trials in Japan (46) as TA-077.

#### *Water-Soluble Nitrosoureas and Chlorozotocin Analogs*

The suggestion that further study of hydrophilic nitrosoureas should lead to more potent, less toxic agents (47) and the discovery that chlorozotocin (fig 9, No. ***21***) was an anticancer agent of reduced bone marrow toxicity (48) accelerated the development of new synthetic methods and synthesis of water-soluble congeners of varied design (32,49–54). It has since been shown, however, that partition coefficients offer little utility in the design of nitrosoureas with greater selectivity owing to the inseparability of antitumor activity and toxicity (1). Furthermore, partition coefficients, which sometimes change as a result of metabolism as with CCNU and semustine (MeCCNU) (55), share importance with other factors such as alkylating activity, stability, and perhaps carbamoylating activity (10).

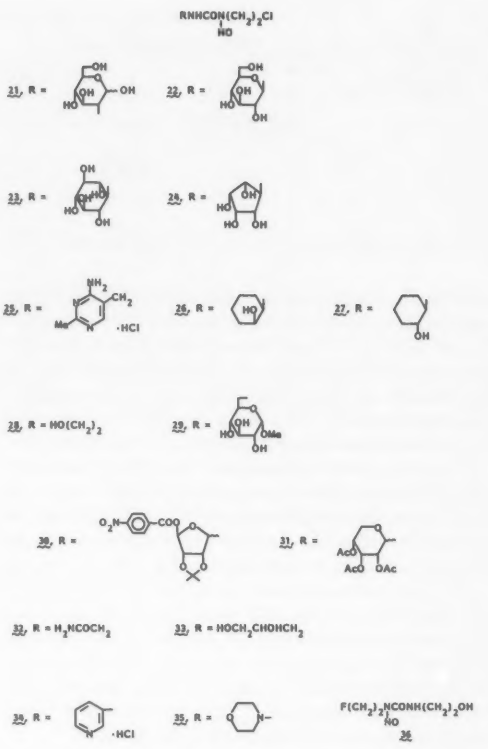



FIGURE 9.

Speculation that the marrow-sparing properties of chlorozotocin were related to water-solubility and low carbamoylating activity due to intracarbamoylation of hydroxyl groups was dispelled in a comparison of seven water-soluble nitrosoureas (fig 9, Nos. ***21–27***) and lipophilic CCNU (56). Only chlorozotocin and GANU (***22***; 57) were nonmyelosuppressive in mice when administered at an LD10 dose, but this property was clearly not mediated by water-solubility per se. The *scyllo*-inositol derivative ***23*** (57,58), the cyclopentanetetrol ***24*** (59), and the self-carbamoylating ACNU (***25***; 60,61), all three water-soluble, showed marrow toxicity comparable to the lipophilic CCNU, as did the more polar hydroxylated, minor metabolites of CCNU (***26,27***; 62). Hence, relative myelo-

toxicity did not correlate with alkylating activity, carbamoylating activity, and hydrophilicity. The latter conclusion was also supported by a subsequent comparison that included the *N'*-(2-hydroxyethyl) congener HECNU (fig 9, No. ***28***; 63). The decreased myelotoxicity of chlorozotocin and GANU appears to be largely mediated by a glucose carrier group and may be related to the observation that their in vitro alkylations of DNA favored L1210 leukemia cells over murine bone marrow cells, whereas the reverse was true for myelotoxic CCNU and ACNU (64). Furthermore, chlorozotocin and GANU preferentially alkylated sites on bone marrow chromatin different from those preferred by CCNU and ACNU (65).

Four water-soluble nitrosoureas are in clinical trials in Japan (46): ACNU (***25***), GANU (***22***), MCNU (fig 9, No. ***29***), and TA-077 (***20***). The clinical selection of ACNU, the first of these to undergo trials, was based on superior activity against L1210 leukemia by either parenteral or oral administration. Being the hydrochloride of a weak base, ACNU is highly soluble at pH 3.3, but much less so at pH 6.8. Although readily administered in solution, ACNU behaves like a lipid-soluble nitrosourea in vivo and has activity against experimental brain tumors (66).

GANU and MCNU are relatives of chlorozotocin, but GANU was somewhat more myelotoxic than chlorozotocin and less so than MCNU. GANU was as active against L1210 leukemia as chlorozotocin by either the ip or iv route, but was also active orally, while chlorozotocin was not significantly so (67). MCNU showed superior activity against L1210 leukemia and Lewis lung carcinoma when compared to chlorozotocin and GANU and showed comparable activity to ACNU and semustine (68,69). Preclinical toxicology predicted gastrointestinal and hematologic toxic effects for both GANU and MCNU and, in addition, hepatotoxicity and nephrytoxicity for GANU (69). Pretreatment of mice with a relatively large amount of sugar alcohol (mannitol, sorbitol, galactitol, or inositol) selectively reduced the acute lethal toxicity of MCNU without reducing its antitumor activity, an observation that was extended to GANU and ACNU as well as to semustine (70). The protection against MCNU toxicity was accompanied by a reduction of both weight loss and myelosuppression.

A series of ribose derivatives (71) afforded two that have found their way into clinical use in France: RFCNU (fig 9, No. ***30***) and RPCNU (fig 9, No. ***31***). Although *O*-substitution rendered these agents water-insoluble, they have been compared extensively with water-soluble nitrosoureas such as chlorozotocin and HECNU. These comparisons revealed no correlation between oncostatic activity against several murine tumors and chemical parameters such as alkylating activity and carbamoylating activity; toxic effects as reflected by LD50s in L1210 leukemia-bearing mice ranged from 25 mg/kg for HECNU to 90 mg/kg for RFCNU. All effected cures of L1210 leukemia, but RFCNU had the widest maximal effective dose range (72). RPCNU was more hematotoxic than RFCNU and chlorozotocin in studies with bone marrow, spleen, and peripheral blood of mice. RFCNU and chlorozotocin had no effect on peripheral blood and moderately decreased colony-forming units in the spleen. Favorable results after repeated injections led to the conclusion that RFCNU belongs to the class of nitrosoureas having low cumulative hematotoxicity (73). Further, RFCNU, in comparison with RPCNU and other sugar derivatives in a hemolytic test in mice, was judged not to be immunosuppressive whether administered before or after the antigen (74).

The above-mentioned HECNU (***28***) emerged with cytostatic activity superior to BCNU from a homologous series of water-soluble nitrosoureas made possible by a synthesis based on the intermediacy of (2-chloroethyl)nitrosocarbamic azide (50). The subsequently expanded list of new products included water-soluble congeners such as acetamido-CNU (fig 9, No. ***32***), dihydroxypropyl-CNU (fig 9, No. ***33***), pyridyl-3-CNU hydrochloride (fig 9, No. ***34***), and the aforementioned morpholine derivative ***5*** (30,75). HECNU is about 30 times more soluble in water than BCNU, yet retains lipophilic properties, crossing the blood-brain barrier more effectively than BCNU (as judged by its 100% cure rate of ic implanted late L5222 leukemia). Similar superiority over BCNU and chlorozotocin was demonstrated against both ip and ic L1210 leukemia and against murine ependymoblastoma. In fact, in comparisons with BCNU and other nitrosoureas, HECNU was either as active as, or more active than, the other most active nitrosourea in nine of 11 rodent tumor models (53,75). The toxic effects of HECNU were reduced by pretreatment with disulfiram without affecting its antitumor potency (76).

In other comparative studies (53,75), acetamido-CNU (***32***) was the most active of the hydrophilic open-chain analogs against L5222 leukemia, achieving cures over a wide dose range, but was somewhat more toxic than HECNU or BCNU. Modifications of the 2-hydroxyethyl group of HECNU by insertion of one or two methylene groups strongly reduced activity against both Walker 256 carcinosarcoma and L5222 leukemia as did the dihydroxypropyl group in ***33*** against the latter. The water-soluble pyridyl-3-CNU hydrochloride (***34***) was very toxic and lacked activity against L5222 leukemia. The derivatization of morpholine as in morpholino-CNU (fig 9, No. ***35***) afforded a highly soluble nitrososemicarbazide, which was highly active against L5222 leukemia, equaled cyclophosphamide in effectiveness against Yoshida sarcoma implanted in the colon of the rat, and surpassed all other nitrosoureas tested against this tumor. HEFNU (fig 9, No. ***36***), the fluoro analog of HECNU, proved to be much more toxic than HECNU and was relatively ineffective against L5222 leukemia (53).

It is difficult to differentiate highly active nitrosoureas on the basis of antitumor test data in rodents and to extrapolate such data to the clinical situation, but significant differences among the clinical nitrosoureas were re-

vealed by long-term toxicity and carcinogenicity tests in rats (53,75). Such tests also showed that the clinical candidate HECNU was less toxic and less carcinogenic than BCNU, while its antitumor activity was superior. A selection of nitrosoureas on the basis of such results would give preference to semustine, with HECNU the best among the newer water-soluble congeners. On the other hand, the highly active morpholino-CNU could not be considered for clinical trials in view of its high long-term toxicity and carcinogenicity.

#### *Nitrosoureas With Special Carrier Groups*

Numerous evaluations of structural modifications among nitrosoureas have tended to standardize the requirement of a 2-chloroethyl (or a 2-fluoroethyl) group on the nitrosated nitrogen for optimal activity against murine leukemia (1,25). One exception to the rule, however, has emerged in the *N,N''*-bis)nitrosourea) EBNU (fig 10, No. *37*), which effected some 30-day cures of L1210 leukemia with three dose schedules in a comparison with BCNU and was by far the most effective of polymethylene series that included bis(nitrosoguanidines) and bis(nitroso-*p*-toluenesulfonamides) (77). The corresponding bis[*N'*-(2-chloroethyl)-*N*-nitrosourea] *38* (fig 10, No. *38*) was inactive against L1210 leukemia (78), whereas the bis[*N'*-(2-chloroethyl)-*N'*-nitrosourea] *39* (fig 10, No. *39*) and its polymethylene homologs with potential bifunctional carbamoylating activity were highly active against L5222 leukemia (50).

The inactivities of *N'*-cyclohexyl-*N*-(2-hydroxyethyl)-*N*-nitrosourea (fig 10, No. *40*; 78) and iso-CCNU (fig 10, No. *41*; 79) against L1210 leukemia were not surprising and provided further support for advocacy of the 2-chloroethyl group. Crowding of the nitroso group in *41* imitates that in the dicyclohexyl congener DCyNU (fig 10, No. *42*), a nonalkylating but strongly carbamoylating nitrosourea of short half-life (80) that was found to be both an inhibitor of DNA repair (11) and a radiation synergist (12) in vitro. The inactivity of DCyNU against L1210 leukemia was not unexpected (1). These observations engendered the synthesis of a number of related compounds among which the dicyclopropyl congener *43* (fig 10, No. *43*) had the shortest half-life under simulated physiological conditions and the highest carbamoylating activity (81).

An attempt to modify the alkylating portion of CCNU, for example, so as to increase the extent of DNA alkylation and crosslinking, succeeded in the case of the *N*-[[2-(2-chloroethyl)thio]ethyl] analog (fig 10, No. *44*), but this effect was accompanied by only moderate activity against L1210 leukemia (82). Most modifications, however, leave the 2-chloroethyl group intact and concentrate on the *N'*-substituent, which produces the carbamoylating moiety and determines variations in solubility, stability, metabolism, transport, and the like. CNCC, a mixture of disulfide-nitrosoureas (fig 10, Nos. *45* and *46*) derived from cystamine, was expected to undergo in vivo reduction and a biotransformation of the resulting thiol (72). These possibly distinguishing features seem to

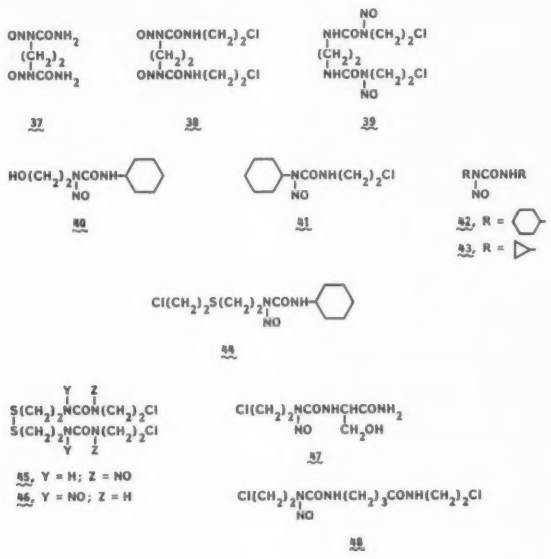



FIGURE 10.

have been beneficial, since ***45*** and ***46*** not only showed favorable activity against several murine tumors but also had the largest maximally effective dose range in comparison with chlorozotocin, RFCNU, RPCNU, and HECNU, and, with RFCNU, was less acutely toxic than the others. Furthermore, the mixture was more effective than pure ***45***.

Extensive efforts to achieve greater chemotherapeutic activity and lower toxicity of nitrosoureas have been directed toward the incorporation, as *N'*-substituents, of moieties derived from amino acids (58,83); amino acid esters (84); amides (32); *N*-(2-chloroethyl)amides (33); and aminocarbohydrate-amino acid derivatives (85). A number of these congeners, for example, the serinamide ***47*** (fig 10, No. ***47***), the γ-aminobutyramide ***48*** (fig 10, No. ***48***), and the aforementioned sarcosinamides ***8*** and ***10***, were highly active against L1210 leukemia, but nothing in their evaluations suggested that biological properties of L-amino acids such as active transport into mammalian tissues played a special role in the observed results. A similar conclusion was reached in the case of D-glucosamine-derived chlorozotocin. Since L-glucose is not actively transported in mammalian tissues, the possible role of D-glucose-mediated transport of chlorozotocin was tested in a comparison with L-chlorozotocin (fig 11, No. ***49***) and found not to be evident; no significant difference was observed in antitumor activity or marrow-sparing effect (86).

Other attempts to improve the pharmacological properties of nitrosoureas by combining them with nucleosides as carriers with possible alteration of metabolism or uptake resulted in what was termed a new class of nitrosoureas, of which 3′-CTNU (fig 11, No. ***50***) and 5′-CTNU (fig 11, No. ***51***) are representatives (87). These are distinguished by the prevention and reversal of in vitro cell growth inhibition caused by 3′-CTNU with thymine, deoxyuridine, or deoxycytidine, which did not decrease that caused by 5′-CTNU or BCNU (88). The coadministration of thymine with 3′-CTNU prevented lethality otherwise produced in non-tumor-bearing mice, but did not affect the anticancer activity against L1210 leukemia and P388 as evidenced by long-term survivals with and without added thymine (89). 5′-CTNU and its deoxyuridine counterpart ***52*** (fig 11, No. ***52***) were also synthesized in a study of various reactive groups that were attached to the 5′-position of nucleosides in an effort to obtain active site-directed enzyme inhibitors (90,91). These compounds were active against P388 leukemia, with ***52*** effecting some 30-day survivals. Several nitrosoureidonucleosides, mostly *N*-methyl-*N*-nitroso- and *N*-cyclohexyl-*N'*-nitrosoureas and exemplified by the inosine derivative ***53*** (fig 11, No. ***53***) and the adenosine derivative ***54*** (fig 11, No. ***54***), were also prepared as potential inhibitors of nucleotide biosynthesis and found to have only a low level of

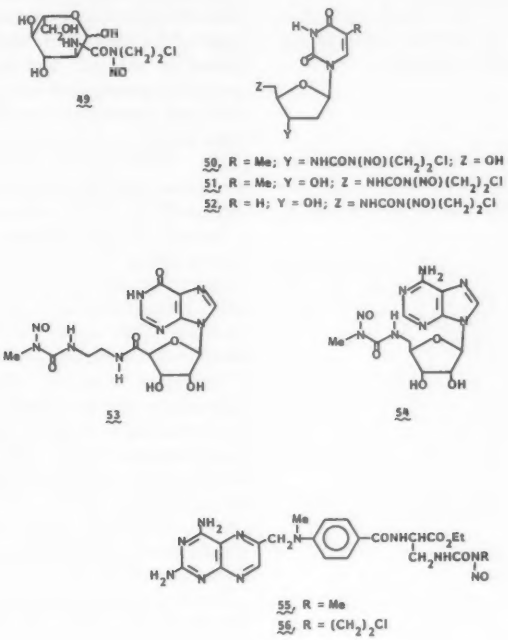



FIGURE 11.

biological activity, which was attributable to unexpected stability, half-lives being observed in hours instead of minutes (92,93).

Methotrexate analogs containing *N*-methyl-*N*-nitrosoureido and *N*-(2-chloroethyl)-*N*-nitrosoureido groups as in compounds ***55*** and ***56*** (fig 11, Nos. ***55*** and ***56***), respectively, have been synthesized for evaluation as antifolates. They have shown appreciable in vivo activity against L1210 leukemia, but this activity did not appear to be attributable to inhibition of dihydrofolate reductase (94).

Nitrosoureas (fig 12, Nos. ***57*** and ***58***) derived from 8-amino-6-methylergoline, an ergot alkaloid, and 17$\alpha$-(aminomethyl)-3,17$\beta$-dihydroxyestra-1,3,5(10)-triene, a steroidal hormone, were, among others, designed to reach tumors of specific organs by special carrier groups. The alkaloid derivative ***57*** had activity against L1210 leukemia in mice, but only moderate prolactin-inhibiting activity in rats; an ergolinylnitrosourea with good prolactin-inhibiting activity might be expected to show specificity for a tumor located in the pituitary gland (95). The steroidal derivative ***58*** showed significant inhibitory activity against a rat mammary tumor, but so did the nonsteroidal CCNU (96). Similar activity had previously been noted with the 3$\beta$-hydroxy-5$\alpha$-androstan-17$\alpha$-yl congener (97). The nitrosourea ***59*** (fig 12, No. ***59***) utilizing phensuximide as carrier and prepared as a potential central nervous system antitumor agent was active against an ic ependymoblastoma and also L1210 leukemia, but was not as active as semustine or BCNU (98). Verifying that a special carrier group functions as designed constitutes a general problem with this group of nitrosoureas.

## Toxicity

Most of the preclinical toxicity data on a vast majority of the nitrosoureas are simply lethal toxicity (LD10) determinations (20) or in many cases, less precisely,

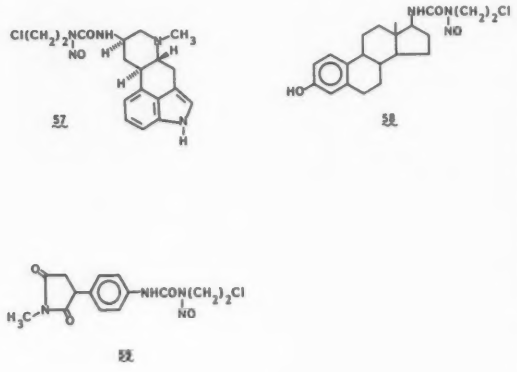



FIGURE 12.

optimal dose values (99). The myelosuppression observed clinically (22) led to a detailed study of this specific toxicity in rodents and to attempts to develop less myelosuppressive agents. Thus, chlorozotocin tetraacetate (100) and other sugar-containing nitrosoureas were shown to be less marrow toxic than the clinically used BCNU and CCNU (48,67,101-107). Unfortunately, although curative in the L1210 leukemia systems, chlorozotocin (108), and presumably its relatives, is much less active or inactive against a spectrum of solid tumors (table 5). The studies referred to above also showed that there is no correlation of myelosuppression with carbamoylating activity (65,105-107), even though carbamoylation was shown to interfere with the DNA repair process (11,12). At the same time the myelosuppressive nitrosoureas have a low ratio of alkylation (L1210:bone marrow DNA) (about 0.6), compared to the marrow-sparing compounds (1.3) (65,101). Furthermore, the myelosuppressive compounds alkylate nucleosome core particles, whereas the other alkylates internucleosomal linker regions of bone marrow chromatin (65).

A detailed study of the toxicity of semustine in monkeys and dogs showed that fatal doses produced severe depression of hematopoietic activity and injury to the mucosa of the gastrointestinal tract in both species. Nephrotoxicity, stemming from primary injury to the proximal convoluted tubules, was a prominent feature of the fatal reactions of the rhesus monkey. Injury to the lung parenchyma of serious dimensions and a significant level of hepatotoxicity in the dog were produced by lethal doses of the drug (109). Nephrotoxicity has also been observed in the rat, and further studies showed that semustine metabolites and/or degradation products preferentially accumulated in the kidney, but the molecular basis for this induced nephropathy is not yet understood (110). Chlorozotocin was more nephrotoxic than semustine, and the nephropathy produced by both drugs appears to be related to their alkylating activity (111).

Interest in the 2-fluoroethylnitrosoureas has been stimulated by the finding that, although the activity of these compounds is comparable to that of the 2-chloroethyl compounds (table 6), the fluoroethyl compounds crosslink DNA poorly (14,15), a result of the greater stability of the carbon-fluorine bond. In addition, the log *P* values are about 0.6 lower, meaning they are more hydrophilic, and the fluoro analog of CCNU, CFNU, has been reported to be less toxic to the bone marrow (112). An examination of the chemical properties of the fluoro and the chloro compounds shows little difference in half-life, alkylating activity, or carbamoylating activity, the alkylating activity being a measure of the reactivity of the (2-haloethyl)diazotates and the stability of the parent nitrosourea (table 7).

There is, however, a significant difference in the toxicity of the fluoro and the chloro compounds, not in LD10 values, which are greater in some cases for the chloro compounds and in other cases for the fluoro com-

TABLE 5.—Nitrosoureas vs solid tumors

| Compound | Tumor* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Advanced Lewis lung | Colon 6/A | Colon 7/A | Colon 8/A | Colon 26 | Colon 38 | Colon 51 | C3H mammary | B16 melanoma | M5 ovarian tumor |
| BCNU | - | + | ND | + | ND | ND | + | - | - | ND |
| CCNU | ++ | - | ++ | + | ND | - | ND | - | ++ | ND |
| Semustine | +++ | +++ | ++ | +++ | ++++ | +++ | ++ | ++ | +++ | ++++ |
| PCNU | + | ++++ | ++ | ND | ND | ND | ND | - | + | ND |
| Chlorozotocin | - | ND | ND | - | - | ND | + | - | - | ++ |

* ND = not determined.

pounds, but in median day of death, which varies from 6 to 27 days for the chloro compounds and from 2 to 5 days for the fluoro compounds (table 8). This difference suggests fluoroacetate toxicity (4), since a significant amount of the fluoro compounds decomposes to 2-fluoroethanol, which is readily oxidized via alcohol dehydrogenase to fluoroacetate. Sodium acetate in aqueous ethanol, which has been shown to reverse fluoroacetate toxicity in mice, given 10 minutes after a single dose of CFNU raised its LD10 from 40 to 90 mg/kg (table 9), although it had no effect on myelosuppression. Enhancement of its effectiveness against L1210 leukemia could not be demon-

Table 6. N-2-(Haloethyl)-N-nitrosoureas vs. Leukemia L1210 (i.p. $10^5$ cells, i.p. single dose)

| RNHCON(NO)CH$_2$CH$_2$X | | $LD_{10}$, mmol/kg[a] | | $ED_{50}$, mmol/kg[b] | | $LD_{10}/ED_{50}$ | | Log 1/C[c] | | Log P[d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| R | X = | Cl | F | Cl | F | Cl | F | Cl | F | Cl | F |
| 2-haloethyl | | 0.19 | 0.043 | 0.093 | 0.036 | 2.0 | 1.2 | 1.0 | 1.5 | 1.5 | 0.33 |
| cyclohexyl | | 0.16 | 0.21 | 0.13 | 0.16 | 1.3 | 1.2 | 0.80 | 0.89 | 2.8 | 2.2 |
| trans-4-methylcyclohexyl | | 0.17 | 0.19 | 0.073 | 0.11 | 2.3 | 1.7 | 1.1 | 0.97 | 3.3 | 2.7 |
| trans-4-ethylcyclohexyl | | 0.36 | 0.31 | 0.12 | -- | 3.0 | -- | 0.93 | -- | 3.8 | 3.2 |
| 2-norbornyl | | 0.26 | 0.28 | 0.11 | 0.14 | 2.4 | 2.0 | 0.96 | 0.87 | 3.0 | 2.4 |
| 1-adamantyl | | 0.22 | 0.26 | 0.19 | -- | 1.2 | -- | 0.72 | -- | 3.6 | 3.0 |
| trans-4-acetoxycyclohexyl | | 0.15 | 0.13 | 0.062 | 0.065 | 2.4 | 2.0 | 1.2 | 1.2 | 2.6 | 2.0 |
| trans-4-acetoxymethylcyclohexyl | | 0.095 | 0.20 | 0.048 | 0.16 | 2.0 | 1.3 | 1.3 | 0.81 | 3.1 | 2.5 |
| trans-4-carboxycyclohexyl | | 0.10 | 0.21 | 0.060 | -- | 1.7 | -- | 1.2 | -- | -1.1 | -1.7 |
| tetrahydrothiopyran-4-yl | | 0.056 | 0.15 | 0.032 | 0.068 | 1.8 | 2.2 | 1.5 | 1.2 | 2.1 | 1.4 |
| tetrahydrothiopyran-4-yl, S,S-dioxide | | 0.060 | 0.12 | 0.029 | 0.037 | 2.1 | 3.2 | 1.5 | 1.4 | 0.19 | -0.41 |
| m-dithian-5-yl, S,S,S',S'-tetraoxide | | 0.14 | 0.24 | 0.026 | 0.088 | 5.4 | 2.7 | 1.6 | 1.1 | -0.90 | -1.5 |

[a] Lethal $Dose_{10}$.

[b] The dose required to produce 50% long-term survivors.

[c] $C=ED_{50}$.

[d] P=Partition coefficient, octanol/water.

Table 7. Some Properties of the N-(2-Haloethyl)-N-nitrosoureas[a, b]

| RNHCON(NO)R X = | $T_{\frac{1}{2}}$ | | Alkylating Activity | | Carbamoylating Activity | |
|---|---|---|---|---|---|---|
| R X | Cl | F | Cl | F | Cl | F |
| cyclohexyl | 100 | 128 | 100 | 123 | 100 | 117 |
| trans-4-methylcyclohexyl | 100 | 111 | 100 | 117 | 91 | 117 |
| trans-4-ethylcyclohexyl | 102 | 104 | 81 | 109 | 72 | 76 |
| m-dithian-5-yl | 62 | 64 | 350 | 403 | 33 | 32 |
| tetrahydrothiopyranyl-4-yl | 42 | 36 | 456 | > 577 | 27 | 26 |
| m-dithian-5-yl, S,S,S',S'-tetraoxide | 15 | 17 | > 577 | > 577 | 0 | 0 |
| 3-thiochroman-4-yl | 116 | 113 | 256 | 354 | 37 | 41 |
| 2-haloethyl | 81 | --- | 266 | 242 | 68 | 47 |

[a]Expressed as % of the values for CCNU. [b]See footnotes to Table 2.

Table 8. Toxicity of Haloethylnitrosoureas

| RNHCON(NO)R X = | Single Dose $LD_{10}$ (mg/kg) | | Multiple of $LD_{10}$, Range | | % Deaths | | Median Day of Death | |
|---|---|---|---|---|---|---|---|---|
| R X | Cl | F | Cl | F | Cl | F | Cl | F |
| 2-haloethyl | 41 | 8 | 1.1 - 2.4 | 1.3 - 3.2 | 30 - 100 | 40 - 100 | 6 - 10 | 2 - 4 |
| cyclohexyl | 57 | 34 | 1.0 - 2.9 | 1.0 - 3.0 | 10 - 100 | 30 - 100 | 8 - 24 | 2 - 3 |
| trans-4-methylcyclohexyl | 44 | 42 | 1.3 - 3.3 | 1.4 - 3.1 | 30 - 100 | 30 - 100 | 7 - 27 | 2 - 3 |
| trans-4-ethylcyclohexyl | 94 | 75 | 1.6 - 4.0 | 1.2 - 5.0 | 40 - 100 | 10 - 100 | 6 - 7 | 2 - 5 |
| trans-4-carboxycyclohexyl | 27 | 56 | 1.1 - 3.4 | 1.2 - 1.8 | 60 - 100 | 30 - 80 | 7 - 22 | 4 - 4 |
| cis-4-carboxycyclohexyl | 26 | 29 | 1.2 - 2.4 | 1.6 - 3.4 | 30 - 100 | 40 - 50 | 7 - 20 | 4 - 4 |
| trans-4-acetoxycyclohexyl | 45 | 36 | 1.7 - 5.6 | 1.0 - 3.4 | 60 - 100 | 10 - 100 | 6 - 16 | 2 - 2 |
| tetrahydrothiopyran-4-yl | 14 | 35 | 1.1 - 3.3 | 1.0 - 2.3 | 0 - 100 | 10 - 100 | 7 - 8 | 3 - 4 |
| m-dithian-5-yl | 23 | 40 | 1.3 - 4.0 | 1.2 - 2.3 | 40 - 100 | 20 - 70 | 6 - 12 | 2 - 4 |
| tetrahydrothiopyran-4-yl, S,S-dioxide | 17 | 32 | 1.2 - 2.7 | 1.2 - 1.8 | 70 - 100 | 35 - 100 | 7 - 12 | 5 - 2 |

strated because the maximum tolerated dose of CFNU alone cured ten of ten animals (table 10). Fluoroacetate toxicity reversal and activity enhancement were observed in the treatment of sc implanted B16 melanoma with the acetate of *N*-(2-fluoroethyl)-*N'*-[*trans*-4-(hydroxymethyl)cyclohexyl]-*N*-nitrosourea and sodium acetate in aqueous ethanol (table 11).

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**Table 9. Reversal of Fluoroacetate Toxicity of N-Cyclohexyl-N'-(2-fluoroethyl)-N'-nitrosourea (CFNU)**

| Single Dose (mg/kg) | Drug Alone | | +NaAc (2 min. post)[a] | | +NaAc (10 min. post)[a] | |
|---|---|---|---|---|---|---|
| | Deaths/ Total | Median Day of Death | Deaths | Median Day of Death | Deaths | Median Day of Death |
| 500 | | | 10/10 | 2 | 10/10 | 2 |
| 400 | | | 10/10 | 2 | 10/10 | 2 |
| 300 | | | 10/10 | 2.5 | 10/10 | 5 |
| 200 | | | 10/10 | 2.5 | 10/10 | 6 |
| 112.5 | 10/10 | 2 | | | | |
| 100 | | | 8/10 | 15 | 1/10 | (29) |
| 75 | 9/10 | 2 | | | | |
| 50 | 9/10 | 2 | 5/10 | 16 | 0/10 | -- |
| 33 | 0/10 | - | | | | |
| 22 | 0/10 | - | | | | |
| | $LD_{10}$ = ca. 40[b] | | $LD_{10}$ = 90 | | $LD_{10}$ = 90 | |

[a]In ethanol-water. No toxicity reversal in water alone. [b]Previously determined $LD_{10}$: 34.

TABLE 10.—Effect of sodium acetate toxicity reversal on the antileukemic activity of CFNU*

| Dose (mg/kg)† | Median day of death | % increase in life span | 30-day survivors ($n$ = 10) |
|---|---|---|---|
| 0 | 7 | — | 0 |
| 50 | 2 | Toxic | 0 |
| 33 | — | — | 10 |
| 22 | 14 | 100 | 1 |
| 300‡ | 2 | Toxic | 0 |
| 200‡ | 6 | -15 | 1 |
| 100‡ | 16.5 | 135 | 8 |
| 50‡ | 19.5 | 178 | 6 |
| 300§ | 2 | Toxic | 0 |
| 200§ | 7 | 0 | 0 |
| 100§ | 19 | 171 | 9 |
| 50§ | 15 | 114 | 9 |

*$10^5$ L1210 leukemia cells implanted iv.

†Single ip injection of drug on Day 1.

‡65 mg of sodium acetate in 0.032 ml of EtOH administered 2 mins after injection of drug.

§Administered 10 mins after injection.

TABLE 11.—Treatment of sc B16 melanoma with the acetate of *trans*-*N*-(2-fluoroethyl)-*N'*-[4-(hydroxymethyl)cyclohexyl]-*N*-nitrosourea*

| Dose (mg/kg) | Toxicity controls, survivors ($n$ = 6) | % increase in life span | T-C (days) |
|---|---|---|---|
| 144 | 0 | -79 | — |
| +NaAc | 2 | 89 | 22 |
| 108 | 1 | -79 | — |
| +NaAc | 6 | 81 | 18 |
| 72 | 6 | -63 | — |
| +NaAc | 6 | 116 | 16 |
| 48 | 4 | 83 | 19 |
| +NaAc | 6 | 62 | 7 |
| 32 | 6 | 56 | 6 |
| +NaAc | 6 | 13 | 3 |

*Treatment on Day 2 after implant followed by sodium acetate in ethanol 10 mins later. LD10 = 42 mg/kg, with sodium acetate at 120 mg/kg.

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# Contributions of Nitrosoureas to Cancer Treatment

**Edith P. Mitchell[1,*] and Philip S. Schein[2,3]**

In 1959, 1-methyl-1-nitroso-3-nitrosoguanidine (MNNG) was found to have reproducible but limited antitumor activity against the murine L1210 leukemia cell line (1). Although it was recognized that the therapeutic potential was insufficient to warrant extensive clinical trial, the findings stimulated the development and intensive evaluation of a wide range of aliphatic nitrosamides as antitumor agents. In a sequence of structure-activity studies, Baker and co-workers at Stanford Research Institute demonstrated that the nitroso group of the MNNG molecule was required for antitumor activity and that substitution of a chloroethyl for the methyl group enhanced its cytotoxicity. Ultimately, these findings led to the synthesis of 1-(2-chloroethyl)-1-nitrosourea (CNU), which showed curative activity for mice bearing the L1210 leukemia (2). Concurrently, Montgomery and colleagues at the Southern Research Institute evaluated a series of compounds with structural similarities to MNNG that were precursors of diazomethane. *N*-Methyl-*N*-nitrosourea (MNU) was found to cross the blood-brain barrier in a cytotoxic concentration, and this finding coupled with evidence of enhanced antitumor activity led to the synthesis of a large series of nitrosoureas with the aim of producing a clinically effective cytotoxic agent.

MNU was found to have greater activity against the ip implanted L1210 leukemia cells than MNNG (3). In addition, it penetrated the blood-brain barrier and increased the lifespan of mice bearing intracranially implanted L1210 cells. This was a significant finding, since the majority of drugs then available for clinical trials did not achieve cytotoxic concentrations within the CNS. It was further recognized at this time that the CNS was a pharmacologic sanctuary for leukemic cells and the site of relapse in one-half of patients in apparent complete remission with acute lymphocytic leukemia. This property was subsequently correlated with the lipid solubility of MNU and led to the development of a series of chloroethyl derivatives including carmustine (BCNU), which showed curative activity against L1210 leukemic cells both ip and intracranially placed in mice (4).

More than 200 congeners of the original MNU molecule were synthesized by Montgomery and Johnson. Of the numbers of nitrosourea compounds synthesized, only one specific structural type, *N*-(2-haloethyl)-*N*-nitrosourea, proved superior to MNU. The 2-iodoethyl compounds lacked antitumor activity, while the 2-bromoethyl derivatives were less effective than the 2-chloroethyl compounds. Although the 2-fluoroethyl compounds showed activity equal to 2-chloroethyl compounds, decomposition to fluoroacetate at pH 7.4 in vitro and in vivo and the resultant central nervous system toxicity in preclinical systems led to a decision to limit their development (5). The original methylnitrosourea (MNU) has now undergone extensive clinical trials in the Soviet Union with demonstrated useful activity in lymphomas and in small cell carcinoma (6). Further studies of alterations of the 2-chloroethyl and the nitrogen group led to the synthesis of lomustine (CCNU), semustine (methyl-CCNU), and PCNU.

The antibiotic streptozocin, a naturally occurring methyl nitrosourea produced by fermentation of *Streptomyces achromogenes*, consists of MNU attached to the $C_2$ position of glucose. Studies showed limited activity against leukemia cell lines, but potent toxicity for the pancreatic beta cell in rodents and higher animal species. It was demonstrated that the diabetogenic activity of streptozocin is mediated by a rapid reduction in pyridine nucleotides in islets of Langerhans and that beta cell destruction and the associated reduction in NAD can be prevented by pharmacological doses of nicotinamide (7). This reduction in NAD is associated with augmented activity of poly-ADP ribose polymerase, a chromatin-associated enzyme that uses NAD as a substrate (8). The glucose (glucosamine) carrier facilitates a more efficient islet cell uptake of streptozocin (9). Streptozocin showed reduced bone marrow toxicity (the dose-limiting toxicity of chloroethyl nitrosoureas in man) and was reported to have some activity against malignant insulinomas and carcinoid tumors, as well as in humans (10). Further studies led to the synthesis of the 2-chloroanalog chlorozotocin, which also showed reduced bone marrow toxicity, but unlike streptozocin was nondiabetogenic (11).

[1]Division of Hematology/Medical Oncology, Department of Medicine, University of Missouri-Columbia School of Medicine.

[2]Clinical Research and Development, Smith, Kline, and French Laboratories, Philadelphia, PA.

[3]Department of Medicine and Pharmacology, University of Pennsylvania School of Medicine, Philadelphia.

[*]*Reprint requests to:* Edith P. Mitchell, MD, Division of Hematology/Medical Oncology, Department of Medicine, University of Missouri-Columbia, School of Medicine, 1 Hospital Dr, Columbia, MO 65212.

The nitrosoureas have shown a broad spectrum of antitumor activity against experimental leukemia and solid tumors. They have established clinical activity for a broad spectrum of human malignancies, including acute lymphocytic leukemia, lymphomas, melanoma, multiple myeloma, gliomas, and gastrointestinal neoplasms.

## Pharmacology

Under physiological conditions the nitrosoureas decompose spontaneously (12,13). The chemical half-lives of individual compounds in phosphate-buffered saline (pH 7.4) vary from 5 minutes for CNU to as long as 2 hours for CCNU (14). In the process of degradation, a number of alkylating moieties are generated, of which an alkyldiazohydroxide precursor and methyl or chloroethyl carbonium ion are considered the most important. Organic isocyanate moieties are also generated and are responsible for carbamoylating intracellular proteins (15,16). In general, amino, carboxyl, sulfhydryl, and phosphate groups are susceptible to alkylation. Carbamoylation of amino acids by the organic isocyanate occurs at sites of "active" hydrogen groups, such as amino groups of lysine or arginine.

In addition to their spontaneous dissociation, the nitrosoureas are also metabolized by the hepatic microsomal mixed function oxidase system to more polar hydroxylated proteins that retain both the cyclohexyl ring structure and the cytotoxic nitrosoureido moiety (17,18). Studies with CCNU indicate that the rate of metabolic hydroxylation exceeds the rate of chemical dissociation and the resulting hydroxylated metabolites are the intermediate precursors of the therapeutic and toxic moieties. The hydroxylated metabolites of CCNU have increased solubility, higher alkylating activities, and in some cases lower carbamoylating activities than CCNU (19,20). Following a 1-hour slow infusion of CCNU, 75% of the plasma drug concentration was in the form of hydroxylated products. Two-thirds appeared as *trans*-4-hydroxy CCNU and one-third as *cis*-4-isomer. In addition to the hydroxylation of the nitrosourea ring structure, studies have shown that both BCNU and MNU are denitrosated by microsomal enzymes (21). All nitrosoureas except ACNU elicit greater mutagenic activity (12%–40%) after incubation with S9 liver microsomal fractions than before (22). Although no correlation between in vitro alkylating activity and mutagenic potential has been demonstrated, results suggest that after S9 activation nitrosoureas possess mutagenic activity equivalent to metabolized 3-methyl-cholanthrene (22). Therefore, this metabolism of nitrosoureas may have relevance in regard to potential carcinogenesis in humans.

## Mechanism of Action

Chemical decomposition generates a series of moieties that alkylate and carbamoylate cellular macromolecules. There is an inverse relationship between the relative activity of each analog and its respective half-life (23,24). The relative alkylating activities of the nitrosoureas have been estimated in reactions with 4-(*P*-nitrobenzyl)pyridine (NBP) (15). Although alkylation is widely accepted as the principal mechanism of nitrosourea antitumor activity, studies have failed to show a linear relationship between in vitro alkylating activity and the chloroethyl antitumor activity for the murine L1210 leukemia (25).

The increased cytotoxic potential of the chloroethyl nitrosoureas when compared with methyl nitrosoureas has been attributed to the ability of the former to cross-link nuclear macromolecules (26–28). This cross-linking is the result of chloroethylation of a nucleophilic site on the first strand followed by displacement of C1 by a nucleophilic site on the same or an opposing DNA strand, forming a cross bridge. Compounds such as MNU and streptozocin can transfer methyl groups to DNA but cannot form cross-links (26). Formation of DNA-DNA or DNA-protein cross-links from an initial monoadduct alkylation is a time-dependent phenomenon (28). It is probable that more efficient removal and repair of alkylated nucleic acids or proteins would reduce the likelihood of cross-link formation, with a presumed concomitant reduction in cytotoxicity. Thus, increased resistance could be a consequence of either (*a*) enhanced repair of alkylated bases or amino acids, or (*b*) reduced conversion of these mono adducts to cross-links (29). In addition, an enhanced ability to repair these may account for drug tolerance.

The correlation of carbamoylating potential of isocyanates generated on decomposition of nitrosoureas to cytotoxicity is unclear. Studies have shown that both chloroethyl (30,31) and methyl (31,32) nitrosoureas carbamoylate a large proportion of nuclear histone and nonhistone proteins (26). In general, the amino, carboxyl, sulfhydryl, and phosphate groups are targets for alkylation, whereas carbamoylation occurs at sites of active hydrogen groups such as ε-amino groups of lysine and arginine. There appears to be no correlation between carbamoylating activity and antitumor activity (15), granulocyte suppression (24), or lethal toxicity (33). Chlorozotocin, ACNU, and streptozocin possess negligible carbamoylating activity but retain antitumor activity. Thus, it is likely that the overall contribution of carbamoylation to cytotoxicity is minor.

The demonstration that isocyanates can inhibit repair of x-irradiation as well as alkylating damage to DNA is particularly important, since inhibition of repair processes could enhance the therapeutic benefit of drugs causing alkylation damage but may also increase their carcinogenic potential and toxicity to normal tissues. Nitrosoureas with high carbamoylating activity delay the repair of x-ray and ultraviolet-irradiation-damaged DNA (34). Kann et al (35) showed that 2-chloroethyl isocyanate, the carbamoylating moiety resulting from decomposition of BCNU, interferes with the ligase step of the excision

process of x-ray damage repair. The effect of 3-β-D-glucopyranosyl-1-methyl-1-nitrosourea (GMNU) and streptozocin on the repair of alkylation changes was reported (33). These drugs have comparable DNA alkylating activity and produce similar numbers of single-strand breaks in L1210 cells in vitro. The rate of repair of L1210 DNA after exposure to GMNU, which has high carbamoylating activity, was significantly slower than after streptozocin, a drug with low carbamoylating activity. However, there was evidence that either the carbamoylating activity or the delay in repair of damage increased cytotoxicity, either in vitro or in vivo.

One of the principal themes of our basic investigations of nitrosoureas has been an attempt to design agents with reduced bone marrow toxicity, but which retain full antitumor activity. This effort has been coupled with studies of sites of drug binding in target and normal tissues.

The optimal in vivo dose of chlorozotocin for L1210 antitumor activity was 48–64 μmols/kg, whereas CCNU required a two to three-fold increased dose, 128 μmols/kg, for comparable maximum therapeutic efficacy. At these doses, chlorozotocin did not reduce the absolute circulating neutrophil count, whereas CCNU reduced the neutrophil count to 37% of control (36). This phenomenon of curative antitumor activity without the necessity for bone marrow toxicity was also evident with a second glucose nitrosourea, GANU, when compared to ACNU and CCNU.

The covalent binding of (ethyl-$^{14}$C) chlorozotocin or CCNU to total cellular and nuclear fractions of L1210 and murine bone marrow cells provided an index of effective drug uptake. In L1210 leukemia cells, 0.1 mM chlorozotocin produced an eightfold increased covalent binding to the cytoplasmic fraction and a fourfold increased alkylation of the nuclear fraction compared to equimolar CCNU. In murine bone marrow cells, the nuclear alkylation was equivalent for the two drugs.

The alkylation of L1210 leukemia and bone marrow DNA by four drugs, the glucose nitrosoureas chlorozotocin and GANU and two potent myelotoxins, ACNU and CCNU, was compared. For L1210 leukemia, 0.1 mM chlorozotocin resulted in 56 pmols of ethyl-1-$^{14}$C group per mg of DNA, 289 pmols/mg of cytoplasmic RNA, and 139 pmols/mg of protein. When compared to results with equimolar concentration of CCNU, this represented a 2.3-fold increased alkylation of DNA and cytoplasmic RNA and a 1.4-fold greater alkylation of proteins. The ratio of quantitative DNA alkylation for L1210:bone marrow was also contrasted. For the two glucose analogs, chlorozotocin and GANU, the ratio was 1.3. In contrast, CCNU and ACNU demonstrated a preferential alkylation of the DNA of bone marrow cells: with CCNU the ratio of alkylation (L1210:bone marrow DNA) was 0.59 and for ACNU it was 0.66.

These murine studies provided a quantitative correlation for the in vivo phenomenon of optimal antitumor activity without the necessity of bone marrow toxicity observed with glucose analogs, ie, increased alkylation of the L1210 nuclear fraction and DNA at equivalent molar concentrations, as well as an advantageous ratio of L1210:bone marrow DNA alkylation.

To provide a qualitative explanation for the reduced bone marrow toxicity of chlorozotocin, we conducted studies of drug binding in regions of L1210 leukemia and bone marrow chromatin. Transcriptionally active chromatin was found to serve as the preferential target for alkylation in murine studies for four nitrosoureas; chlorozotocin, GANU, and the more myelotoxic CCNU and ACNU were compared. Each compound was found to preferentially alkylate DNA with the nucleosomal core particles of L1210 leukemia cell chromatin (37). In contrast, the two nitrosoureas with a glucose carrier (chlorozotocin and GANU) preferentially alkylated the internucleosomal regions of murine bone marrow chromatin, while the preferred targets of CCNU and ACNU were the nucleosome core particles. This work is the first demonstration that members of the same class of alkylating agent have different tissue-specific binding sites within the chromatin structure, and these differences may account in part for the differential sparing of murine bone marrow tissue by the glucose nitrosoureas chlorozotocin and GANU. In addition, in murine bone marrow the more myelotoxic CCNU was found to preferentially alkylate the transcriptionally active region of chromatin when compared with chlorozotocin. Approximately 11% and 7% of marrow DNA was digested by DNase II in cells pretreated with chlorozotocin and CCNU, respectively. Less than 20% of chlorozotocin-derived alkylations were digested, while 40% of CCNU-derived alkylations appeared in the soluble fraction. This tropism of CCNU for template-active murine bone marrow DNA may correlate with the observed severe marrow toxicity of CCNU.

We extended these enzymatic digestion studies to human bone marrow cells, and have completed a detailed study of the factors contributing to the reduced hematologic toxicity of chlorozotocin in human marrow cells (38). After a 2-hour incubation with equimolar ($^{14}$C) chloroethyl chlorozotocin or CCNU, there was equivalent covalent drug binding to whole marrow cells and equivalent quantitative alkylation of DNA (chlorozotocin, 22.8 ± 1.2 pmols/mg of DNA; and CCNU, 22.9 ± 0.5 pmols/mg of DNA). These results are similar to those we reported with murine bone marrow cells, where there was equivalent quantitative alkylation of bone marrow DNA by chlorozotocin and CCNU, but the less myelotoxic chlorozotocin demonstrated an advantageous ratio of L1210:bone marrow DNA alkylation (1.3) when compared to CCNU (0.6).

DNA strand breakage and DNA-DNA and DNA-protein cross-linking was measured by alkaline elution of pooled fractions of elutriated bone marrow, using alkaline elution methodology. The two drugs produced a comparable degree of DNA strand breakage and DNA-protein cross-

linking, as measured by alkaline elution of pooled fractions of elutriated bone marrow. These results were correlated with the equivalent alkylation of human bone marrow DNA by the two drugs. DNA interstrand crosslinking was not found with either drug.

The nucleases DNase II, DNase I, and micrococcal nuclease were utilized to digest specific regions of bone marrow chromatin after treatment with CCNU or chlorozotocin. CCNU alkylations were preferentially in the transcriptionally active regions of human bone marrow chromatin, whereas chlorozotocin predominately alkylated the transcriptionally inactive regions. In cells treated with CCNU, 16.6% ± 4.1% of chromatin was solubilized, and 67.4% ± 8.2% of CCNU alkylations was released after a 10-minute digestion with DNase II. In contrast, in chlorozotocin-treated cells digestion of 27.3% ± 5.2% of the chromatin with DNase II resulted in the release of only 12.8% ± 3.6% of chlorozotocin alkylations. Similar results were obtained with DNase I, an enzyme that preferentially digests transcriptionally active chromatin without preference between nucleosomal core and linker regions. The kinetics of DNase I-limited digestion demonstrated an increased release of CCNU alkylations as compared to chlorozotocin; these data are consistent with preferential alkylation of transcriptionally active regions of bone marrow chromatin by CCNU, as well as nucleosomal core regions.

In summary, despite equivalent quantitative binding to total bone marrow DNA, we found qualitative differences in the alkylation of specific regions of human bone marrow chromatin by chlorozotocin and CCNU. The more myelotoxic CCNU demonstrated a tropism for template-active regions of bone marrow chromatin.

The therapeutic philosophy in the design of the phase I and II clinical trials of chlorozotocin conducted at the Lombardi Center was as follows: define a phase I dose and schedule that produced minimum leukopenia and thrombocytopenia and evaluate that dose in tumors in which nitrosoureas have an established role in therapy (39-41). The dose ultimately selected was 120 mg/m$^2$, which produced levels of intact nitrosourea equivalent to a 150-mg/m$^2$ dose of BCNU. It should be recognized that in equivalent molar concentrations chlorozotocin has the potential to provide twice the alkylating activity of BCNU based on in vitro analyses using the NBP reaction. Chlorozotocin was evaluated in both melanoma and lymphoma and produced response rates equivalent to those described for other chloroethyl nitrosoureas, but without evidence of cumulative or serious bone marrow toxicity. Unfortunately, this approach to clinical development, specifically to validate the laboratory phenomenon of antitumor activity without the necessity of significant myelosuppression, was not recognized by other institutions that evaluated chlorozotocin at so-called "maximum tolerated doses," where this selective effect on tumor could not be appreciated.

## Clinical Therapeutic Activity

The initial phase I trial of BCNU, conducted in 1962, demonstrated clinical activity in lymphomas. Unfortunately, the problem of delayed and cumulative bone marrow toxicity was not appreciated in the initial toxicology studies, and the original schedule of frequent drug administration produced serious and lethal toxicity in patients. In recognition of this inherent treatment-limiting toxicity, studies designed subsequently have employed intermittent schedules of treatment, to permit for full bone marrow recovery between each course. This represents an important dose limitation of this group of chemotherapeutic agents, which complicates the design of combination chemotherapy trials. The most commonly employed dose schedules for the chloroethyl nitrosoureas, when used as single agents in previously untreated patients, include the following: BCNU (150-200 mg/m$^2$ iv every 6-8 weeks); CCNU (100-130 mg/m$^2$ orally every 6-8 weeks); and methyl-CCNU (150-200 mg/m$^2$ orally every 6-8 weeks).

The chloroethyl nitrosoureas have been demonstrated to possess a broad spectrum of activity against human malignancies. The major indications include gliomas, lymphoproliferative diseases, small cell carcinomas of the lung, melanoma, and gastrointestinal cancer (42).

The development of nitrosoureas has increased the efficacy of chemotherapy for the treatment of brain tumors. The use in gliomas represents an attempt to exploit the lipid solubility of most members of this class of antitumor agents. Most of these compounds are nonionized and lipid soluble and, with the capability to cross the blood-brain barrier, penetrate deeply into brain tumor tissue and eventually the intracellular compartment and manifest an antineoplastic effect. The overall reported response rates in the literature for BCNU and CCNU are 45% and 37%, respectively, whereas methyl-CCNU, at 23%, appears to be less active (42).

The first reports of phase II clinical trials utilizing BCNU for the treatment of recurrent malignant tumors demonstrated a remission rate of 50%, with a median duration of response of 6-9 months (43,44). Weir et al (45) reported the results of a controlled clinical trial in patients with recurrent disease. Patients were randomized to receive CCNU (130 mg/m$^2$/day every 6-8 weeks) or radiotherapy (4000-4500 rads) alone or in combination. The median times to progression for CCNU, radiotherapy, and combined treatment were 14, 23, and 31 weeks, with a median survival of 37, 27, and 36 weeks, respectively.

The Brain Tumor Study Group carried out an important controlled multi-institutional clinical trial designed to evaluate nitrosourea chemotherapy in patients with glioblastoma who had undergone a definitive surgical resection (46). Patients were randomized to receive one of four postoperative treatment options: (*a*) BCNU, 80 mg/m$^2$/day on 3 successive days every 6-8 weeks; (*b*) ra-

diation therapy (5000–6000 rads over 6–8 weeks); (*c*) a combination of BCNU plus radiation therapy; and (*d*) best conventional care. An analysis of survival data for patients who received at least 5000 rads of irradiation or two courses of BCNU revealed the following: the median survival for conventional care was 17 weeks; this was contrasted with a 25-week median for BCNU, 37.5-week median for radiation therapy, and 40.5-week median for the combination. All forms of active therapy, including BCNU alone, provided a statistically better survival than conventional postoperative care. The differences between radiation therapy and the combined modality approach were not significant, however. The survival at 18 months for radiation therapy was 9% versus 18% for BCNU plus irradiation or marginal improvement but achieved without appreciable additive toxicity.

Further studies were carried out to investigate more extensively the role of chemotherapy in the treatment of brain tumors (47). Patients were randomized to receive (*a*) radiation alone (6000 rads in 6–7 weeks); (*b*) methyl-CCNU (220 mg/m$^2$ orally once every 6–8 weeks); (*c*) radiotherapy plus methyl-CCNU; or (*d*) radiation therapy plus BCNU (80 mg/m$^2$/day on 3 successive days). The median survival for methyl-CCNU alone was 24 weeks as compared to radiotherapy, 36 weeks; methyl-CCNU and radiotherapy, 42 weeks; and BCNU plus radiotherapy, 52 weeks. Although the additional value of methyl-CCNU or BCNU to radiotherapy was only modest, the results further substantiated the superiority of combined therapy.

Combination chemotherapy utilizing nitrosoureas with other agents has been evaluated in phase II trials. The regimen of CCNU, procarbazine, and vincristine was found to be active against recurrent adult malignant glioma (48,49) and medulloblastoma (50), achieving a response rate of 60% for a median of 9–10 months. With a revised drug schedule utilizing these same drugs, Levin et al (51) reported a response rate of 84%, with a median time to progression of approximately 28 weeks in previously untreated patients.

Because brain tumors seldom metastasize to the bone marrow, the reported response rates to conventional chemotherapy approaches have prompted investigations of high-dose therapy and autologous bone marrow transplantation (ABMT). Hochberg et al (52) reported the first study using high-dose BCNU and ABMT. During relapse following radiation therapy, 11 patients with glioblastoma multiforme were treated with 600–1400 mg/m$^2$ of BCNU followed by autologous marrow rescue. Eight of these patients experienced a diminution in contrast enhancement and cerebral edema as evidenced by computerized tomographic scan. Complete responses, prolonged remissions, and clinical improvement were noted in a small number of patients. Although these results were not superior to those with standard doses of BCNU, further studies were encouraged. Carella et al (53) reported results of treatment of four patients with glioblastoma multiforme in remission following primary treatment with surgery, radiotherapy, or a combination of both. Therapy consisted of BCNU at a dose of 800 mg/m$^2$ followed by ABMT. One patient remained in remission 12 months after therapy; however, no conclusion can be drawn from this limited study. Phillips et al (54) described a study in which patients with glioblastoma multiforme received BCNU (600–1350 mg/m$^2$) and ABMT. Of ten evaluable patients, one with extensive disease and one treated as adjuvant after resection remain in complete remission at 249 and 179 weeks, respectively. Further carefully designed clinical studies should determine the roles of high-dose chemotherapy with ABMT.

The principal indication for nitrosourea therapy in lymphoproliferative disorders is Hodgkin's disease. Five independent clinical trials of BCNU chemotherapy, following relapse from previous therapy, have shown an overall objective response rate of 47%, with a range of 34%–55% (55–57). Response usually occurred early, within 2 weeks, with a median duration of 4 months. The Cancer and Leukemia Group B (CALGB) has tested the activity of three chloroethyl nitrosoureas in patients with advanced previously treated Hodgkin's disease (58,59). In the initial trial, BCNU (200 mg/m$^2$) was compared with CCNU (100 mg/m$^2$); the latter drug produced a 69% objective response which was superior to the 36% achieved with BCNU. Methyl-CCNU proved inferior to CCNU in a subsequent clinical trial, having produced only a 15% response compared to a response of 42% for CCNU. From these studies it was concluded that as a single agent CCNU displayed the greatest activity in the treatment of Hodgkin's disease.

The CALGB subsequently initiated a comparative trial of CCNU and mechlorethamine in stage III-B and IV disease (59). Therapy was administered with two four-drug regimens containing CCNU (cyclophosphamide, vinblastine, procarbazine, and prednisone; and cyclophosphamide, vincristine, procarbazine, and prednisone) and two combinations that were otherwise identical [mechlorethamine, vinblastine, procarbazine, and prednisone; and mechlorethamine, vincristine, procarbazine, and prednisone (MOPP)], except that mechlorethamine was the alkylating drug. The CCNU-containing combinations have produced complete remissions greater than or equal to MOPP. This included previously untreated patients as well as those with stage III-A disease having relapsed after primary radiation therapy.

The Southeastern Cancer Study Group has used BCNU in a multidrug regimen with cyclophosphamide, vinblastine, and procarbazine. Patients achieving a complete response at the end of six monthly cycles of carmustine, cyclophosphamide, vinblastine, procarbazine, and prednisone (BCVPP) were randomized to receive (*a*) six additional cycles of BCVPP, (*b*) six cycles of standard MOPP, or (*c*) no further therapy. Using this regimen, 324 evaluable patients with advanced Hodgkin's disease were treated with BCVPP. Complete responses were observed

in 122 (56%) of 180 previously untreated patients (60). No difference in the duration of remission or survival was evident when either maintenance arm was compared to those patients receiving no further therapy. These results showed evidence that BCVPP was an effective alternative to MOPP in the management of advanced Hodgkin's disease. Complete and overall response rates and durations of response were equivalent to those observed with MOPP. Furthermore, BCVPP required fewer outpatient visits and demonstrated less gastrointestinal side effects.

It is therefore apparent that the chloroethyl nitrosoureas have an established role in the treatment of advanced Hodgkin's disease, although none of the regimens utilizing nitrosoureas in combination chemotherapy proved to be superior to MOPP.

Single-agent activity has been demonstrated for streptozocin in patients with advanced-stage and heavily pretreated Hodgkin's disease. In phase II trials involving 16 patients, 44% achieved partial remission without evidence of myelosuppression. No cross-resistance with other nitrosoureas was noted. Of eight patients whose disease was refractory to BCNU and/or CCNU, three achieved partial response and one achieved complete remission with streptozocin. In another patient where streptozocin was used first, a subsequent response was obtained utilizing CCNU.

The nitrosoureas have limited single-agent activity for small cell carcinoma of the lung (SCCL). The reported response rate for CCNU is 14% and for both BCNU and methyl-CCNU is 26% (61-63). Both streptozocin (64) and chlorozotocin (65) lacked activity in SCCL in phase II trials. Because significantly improved survival was demonstrated with CCNU when compared to placebo in extensive-stage patients studied by the Veterans Administration Lung Cancer Study Group (66), this nitrosourea became the agent of choice in SCCL.

Hansen et al (67) conducted a randomized study to determine the value of adding CCNU to the combination of cytarabine and methotrexate. Higher response rates and superior survival were reported with this three-drug nitrosourea combination. Cohen et al (68) reported results of a randomized trial designed to determine whether the intensity of therapy using a three-drug combination of CCNU, cytarabine, and methotrexate (CMC) was important. Patients were randomized to receive either low-dose CMC therapy consisting of 500 mg/m$^2$ of cytarabine, 10 mg/m$^2$ of methotrexate, and 50 mg/m$^2$ of CCNU or high-dose CMC therapy consisting of one cycle of 1000 mg/m$^2$ of cytarabine, 15 mg/m$^2$ of methotrexate, and 100 mg/m$^2$ of CCNU followed by the lower dose schedule. Patients receiving the high-dose therapy had longer median survival times and higher response rates, with a total complete plus partial response rate of 96% when compared to the low-dose regimen (complete + partial response rate, 44%). Complete responses were only observed in those patients receiving the high-dose regimen, while in the maximum tumor, response was observed within the first 6 weeks of therapy. Further studies by this group demonstrated a high response rate to the combination of doxorubicin, procarbazine, and vincristine in patients with evidence of disease progression in the nitrosourea-containing regimen (69).

Clinical trials utilizing alternating non-cross-resistant nitrosoureas-based chemotherapy combinations were introduced. Cohen et al (70) reported an increase in complete response rate from 30% at 6 weeks to 48% at 12 weeks, however, with no impact on median or disease-free survival. Aisner et al (71) found no influence on response rates, remission duration, or survival when an alternating non-cross-resistant regimen of CCNU, methotrexate, procarbazine, and vincristine was compared to continuous administration of a single three-drug regimen of cytarabine, doxorubicin, and etoposide. Other combinations showed similar findings. Daniels et al (72), however, reported results of a clinical trial in which patients with SCCL were randomized to receive alternating or sequential combination chemotherapy with the three-drug regimen of etoposide, doxorubicin, and methotrexate and a four-drug regimen consisting of cyclophosphamide, vincristine, procarbazine, and CCNU. The alternating arm was superior with respect to rate of complete remission, median disease-free survival, and median survival.

One of the rationales for the incorporation of a lipid-soluble nitrosourea in a drug combination for SCCL has been an attempt to prevent CNS relapse. The overall incidence of brain, spinal cord, or leptomeningeal involvement by SCCL is estimated to be 40% and as high as 60% in some series. Clinical trials employing nitrosoureas have clearly demonstrated that these agents make no impact on the incidence of CNS metastases (73).

The activity of the nitrosoureas in gastrointestinal cancers has been investigated. They have demonstrated only modest activity as single agents with response rates of 8%-18% in gastric cancer, 0%-9% in pancreatic cancer, and 9%-13% in advanced colorectal carcinoma. Nevertheless, in some studies combinations of nitrosoureas and 5-FU have resulted in response rates that exceeded those achieved with 5-FU alone. In a randomized trial, Kovach et al (74) reported a 41% objective response rate with 5-FU plus BCNU, 29% for 5-FU alone, and 17% for BCNU alone in patients with advanced gastric cancer. Significant improvement in survival at the 18-month follow-up was observed in patients treated with the combination (27% vs 7%). In another randomized trial by the Eastern Cooperative Oncology Group (ECOG) (75), patients were treated either with methyl-CCNU alone or 5-FU plus methyl-CCNU, either with or without a cyclophosphamide induction course. The combination produced a response rate of 40% compared to 80% with methyl-CCNU alone. The median survival of patients treated with the combination was 20 weeks, whereas patients treated with the single agent lived a median of 13 weeks. Thus, both the response rate and survival in patients treated

with 5-FU and methyl-CCNU proved superior to the other three (76). Activity for the chemotherapy combination of methyl-CCNU, doxorubicin, and 5-FU has been demonstrated (77-79).

The combination of BCNU plus 5-FU has been studied in pancreatic carcinoma. A greater response rate (33%) was observed with the combination than with either drug used alone, but with no apparent impact on survival (74). By contrast, streptozocin, combined with mitomycin and 5-FU, has produced a 43% response rate, with improved survival in responding patients (79).

Combinations of nitrosoureas with other agents have been extensively studied in colorectal cancer. Falkson et al (80) reported a 43% response rate for the combination of BCNU, dacarbazine (DTIC), and vincristine as compared to 25% for 5-FU alone. Baker et al (81) and Moertel et al (82) reported similar response rates for the combination of methyl-CCNU, 5-FU, and vincristine. Kemeny et al (83) reported a total response rate of 52% in patients treated with the combination of methyl-CCNU, streptozocin, 5-FU, and vincristine. Further testing by other investigators has not confirmed these findings. The use of adjuvant chemotherapy with nitrosoureas in surgically resected colorectal cancer has shown no substantial differences in relapse rate or overall survival.

In our series of 12 patients with metastatic islet cell carcinoma treated with streptozocin as a single agent, three patients achieved complete clinical disappearance of tumor and hormone production. This included a patient with advanced malignant insulinoma who had an objective response for 1 year after a single iv course at a daily dose of 500 mg/m$^2$ for 5 consecutive days (84). Two patients with "pancreatic cholera" and islet cell carcinoma achieved complete or near complete response to treatment with intra-arterial streptozocin (88). Before therapy, they had stool volumes from 2 to 8 L/day and required 200-300 mEq/day of supplemental potassium. After three to five doses of streptozocin, 1.5 g/m$^2$ at weekly intervals, both stool volume and number and size of hepatic metastases decreased markedly. Partial remissions, averaging 4 months in duration, have been achieved in three additional patients with tumors that produced insulin, gastrin, or serotonin. Our results are in general agreement with data compiled by the Cancer Therapy Evaluation Branch of the National Cancer Institute (Bethesda, MD), which analyzed the records of 52 patients treated with streptozocin (89). In patients with functioning tumors, 60% were reported to have experienced a lessening in severity of hypoglycemia or hyperinsulinemia, and in 26% of these hormonal parameters returned to normal levels. Three patients in whom the tumor was secreting multiple hormones, insulin, and gastrin, in addition to glucagon in one, have responded. Objective reduction of tumor had been reported in 48% of patients with functioning tumor; 17% were considered to have achieved complete remission. Five of eight patients with islet cell carcinoma without demonstrable hormone secretion also had a reduction in tumor size. The median duration of objective remission was approximately 1 year. The median survival of patients who have responded to streptozocin was 4.5 years, compared to 1.5 years for nonresponders. The latter figure is similar to the median survival recorded prior to the advent of specific chemotherapy. Although streptozocin is quite specific for the beta cell of the animal islet, it is of interest that this drug has proved to be an effective therapy for patients with non-beta-cell neoplasms. In addition to our patients with pancreatic cholera and serotonin secretion, there have been patients with pure gastrin-secretion (Zollinger-Ellison) non-beta-cell carcinoma who have similarly responded to treatment with streptozocin.

Streptozocin is now almost routinely combined with 5-FU for the management of islet cell carcinomas. The ECOG compared the efficacy of single-agent streptozocin with the combination (86). Fifteen of 42 patients (36%) treated with streptozocin responded, compared to a 63% response rate with streptozocin and 5-FU. Although not statistically significant, patients treated with the combination have survival improvement, 26-month median versus 16.5 months with single-agent streptozocin.

Streptozocin also has a role in the management of malignant carcinoid tumors. The ECOG conducted a trial in which streptozocin was combined with 5-FU (87); a 33% response rate was reported for tumors arising in all sites, and with small bowel carcinoids a 41% response was observed, with a median survival of 29 months.

The nitrosoureas have demonstrated a single-agent activity for metastatic malignant melanoma (39,90). The reported objective response rates of 12%-26% are comparable to the activity of DTIC. The advantage of chlorozotocin, CCNU, and methyl-CCNU is that they can be administered orally and with less gastrointestinal toxicity than with DTIC. High-dose BCNU (1050-1200 mg/m$^2$) given over 3 days followed by cryopreserved ABMT may enhance the effectiveness. Fay et al (91) observed five complete and nine partial responses in 30 patients with advanced melanoma. Three of ten patients with brain metastases responded (one complete). Standard doses of BCNU via carotid or vertebral artery infusion showed no effect on brain lesions (92). Attempts to increase response rates by combining nitrosoureas with DTIC or other antineoplastic agents have not resulted in improved effectiveness.

The nitrosoureas are effective in the treatment of plasma cell myeloma; however, they have not been shown to be equal or more effective than melphalan or cyclophosphamide (93). The Southwest Oncology Group found that chemotherapy combinations containing BCNU with melphalan and cyclophosphamide did not significantly change the response rate or survival (94). In contrast, the CALGB reported significant improvement in the response rate in several parameters with the combination of melphalan, cyclophosphamide, BCNU, and prednisone when compared to melphalan and prednisone alone; how-

ever, no improvement in survival of patients was noted (95). Thus, cyclophosphamide and melphalan continue to be the alkylating agents most commonly used in the treatment of plasma cell myeloma.

### Clinical Toxic Effects

The clinical toxicity of the chloroethyl nitrosoureas has been reported (42,96). These drugs produce immediate gastrointestinal side effects and two major dose-limiting toxic effects. Cumulative myelosuppression is more commonly observed with the chloroethyl nitrosoureas and renal tubular damage is observed with the methylnitrosourea, streptozocin. Other chronic toxic effects to the lung and kidneys present following prolonged therapy over many months. CNS toxicity has been encountered in high-dose therapy used in an attempt to increase antitumor efficacy.

Gastrointestinal toxicity manifests as varying degrees of nausea and vomiting and is common to all agents (85). Streptozocin, however, has the greatest emetogenic potential. More than 90% of patients experienced some degree of nausea and vomiting, with protracted vomiting occurring in 10% of patients (84). It usually occurs 2-4 hours after administration and may be decreased with premedication with phenothiazines or other antiemetics; it is usually of short duration.

The most serious toxic effect is myelosuppression, with nadir platelet and wbc counts occurring 4-5 weeks following administration, requiring 1-2 weeks for recovery. For most patients, the thrombocytopenia predominates. After repeated courses of treatment, blood cell count recovery is progressively longer and chronic bone marrow hypoplasia may persist. Accentuation of nitrosourea marrow suppression has been reported with concomitant treatment with cimetidine (97) and theophylline derivatives (98).

Renal tubular damage, resulting in a full Fanconi-like syndrome and renal failure, is the major dose-limiting toxic effect of streptozocin (84). We noted renal abnormalities in 28% of 106 patients treated. Nephrotoxicity was the major contributing factor of death in four of these patients. Hypophosphatemia is usually seen as the earliest abnormality and may appear after only one dose of drug. Acetonuria, aminoaciduria, glycosuria, and hyperchloremia are a part of the syndrome (99). In addition, evidence for glomerular damage with elevations in BUN and creatinine has been reported (89). Renal function may return to normal if the drug is stopped early after abnormalities appear; however, irreversible nephrotoxicity will occur with continued treatment.

Although renal toxicity with chloroethyl nitrosoureas is an uncommon finding, increasing incidence is seen in prolonged therapy, resulting in high cumulative doses (100-104). Harmon et al (100) reported nephrotoxicity in six of 17 patients treated with methyl-CCNU. All patients received total doses > 1500 mg/m$^2$ and none developed renal insufficiency during nitrosourea treatment. During therapy, progressive decrease in the size of the kidneys was noted and continued, despite cessation of therapy. Irreversible renal failure occurred after completion of the 2-year course of treatment. Pathologically, thickening of glomerular basement membranes and tubular atrophy were observed. In a retrospective review of patients treated with methyl-CCNU, Nichols and Moertel (101) reported nephrotoxicity in four of 857 patients. Three of these patients had received cumulative doses > 1700 mg/m$^2$. Fourteen percent of the patients whose total cumulative dose was > 1000 mg/m$^2$ had evidence of renal impairment. Schact et al (104) reported renal dysfunction in six patients treated with BCNU. Total cumulative doses in all were > 1500 mg. The nephrotoxic potential of the nitrosoureas appears to be well-established, with the insidious progressive development of renal dysfunction months to years after cessation of chemotherapy. Initially, the urinalysis and serum BUN and creatinine may all be normal, with abnormalities detected only by more sophisticated tests such as glomerular filtration rate and renal plasma flow. If therapy with chloroethyl nitrosourea is continued for > 15 months or if the cumulative dose is > 1000 mg/m$^2$, one should suspect the possibility of nephrotoxicity and discontinue therapy if renal size decreases or if substantial diminution of glomerular filtration rate occurs.

Pulmonary toxicity is most frequently associated with BCNU (105-108), although it has been reported with methyl-CCNU (110) and chlorozotocin (111). Large cumulative doses and prolonged administration are associated with increased pulmonary risks. Aronin et al (105) observed pulmonary toxicity in 50% of patients receiving total doses > 1500 mg/m$^2$ of BCNU. Clinically, symptoms of nonproductive cough, dyspnea, and tachypnea were noted. Physical abnormalities were limited to basilar crepitant rales. The roentgenographic findings included normal chest x-rays and bilateral interstitial infiltrates. A restrictive ventilatory defect, with resting hypoxemia and a decreased carbon monoxide diffusing capacity, was observed with pulmonary function studies. Histologically, metaplasia of alveolar epithelium and proliferation of type II pneumocytes and fibroblasts (109) were observed. Pulmonary dysfunction was progressive and corticosteroid treatment offered no benefit. Most patients die due to complications.

Myelodysplastic syndrome as well as acute nonlymphocytic leukemia has been reported with chloroethyl nitrosoureas as single agents (112-116) and combined with other chemotherapeutic agents with and without irradiation. The majority of patients had prolonged myelosuppression after nitrosourea treatment prior to the development of leukemia. Acute myelogenous leukemia following chemotherapy and/or radiotherapy is characterized by a high incidence of trilineage involvement, difficulty in classification, frequent cytogenetic abnormalities, and poor response to antileukemic therapy (115). Michels et

al (115) observed complete remissions in ten of 32 patients with acute myelogenous leukemia or myelodysplastic syndrome. The median survival was 4 months and no significant difference was noted between untreated patients and those treated with antileukemic therapy.

Other rarely reported toxic effects of nitrosourea therapy include encephalopathy, dizziness, ataxia, optic neuritis, and seizures (117,118). Encephalopathy associated with cerebral white matter necrosis at autopsy was reported in four patients after receiving a single treatment of high-dose BCNU (1200 mg/m$^2$) (119). Conjunctival flushing and local venous irritation commonly occur with BCNU. Although streptozocin has acute diabetogenic activity in animals, the normal human beta cells appear to be resistant to this action, with only a rare case of diabetes reported. The acute diabetogenic activity of streptozocin is related to the drug's selective uptake into islets and its ability to depress levels of the pyridine nucleotides NAD and NADH. This activity can be blocked with pharmacological doses of nicotinamide (84).

## CONCLUSION

The nitrosoureas are an important class of clinically active antineoplastic agents. At present, the phenomenon of drug resistance and the described clinical toxic effects are major obstacles in the more successful application of this form of chemotherapy. Further understanding of the molecular mechanism for these processes is prerequisite to overcoming the problems, and to the rational design of new nitrosoureas and other classes of alkylating agents with a greater therapeutic index.

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# Preclinical Selection of New Anthracyclines

**A. M. Casazza**[1,2,*]

One of the most investigated classes of antitumor agents is constituted of anthracyclines. The most well-known anthracycline is doxorubicin (1,2), which has become widely integrated into cancer chemotherapy since its introduction into clinical use (3). So far, 16 anthracyclines[3] have been introduced into clinical trials (4). They represent the top of an iceberg constituted by hundreds of new congeners of this class, which have been obtained by chemical synthesis or by fermentation, and have been investigated to identify compounds superior to the parent drugs in terms of increased therapeutic effectiveness, reduced toxicity, or both. High efforts have been done world-wide in this field, not only by the pharmaceutical industry, but also by academic institutions and governmental entities, and several new anthracyclines have reached approval by health authorities for clinical treatment. Clinical feedback into experimental drug development of new anthracyclines is just starting, and we can say that clinical studies seem to be, in most cases, confirmatory of preclinical expectations. However, several very important points remain obscure. This review represents an effort to consider and discuss some of these points.

## Mechanism of Action

Structure-activity as well as structure-toxicity relationships should be based on the knowledge of the mechanisms of action and of toxicity of the drug considered. In the case of anthracyclines, such knowledge is still lacking. The extensive studies carried out in the past 20 years on this important class of antitumor agents have suggested that anthracyclines can act through several mechanisms (table 1).

Binding to DNA was the first biochemical action recognized for anthracyclines (5). Studies carried out examining a high number of compounds have recently confirmed that there is a general relationship between the ability of binding to DNA and the cytotoxicity of anthracyclines on in vitro-cultured cells, as well as the general toxicity in tumored mice, as established by evaluation of the maximal tolerated dose (6). For homogeneous series of anthracyclines, it was possible to establish a linear inverted correlation between affinity of binding to DNA and ID50 on HeLa cells (fig 1) (7). However, some anthracyclines escaped this rule. In this regard, it is interesting to recall the work done by Chen et al (8) and Fico et al (9) in the field of aminoacridines. Bis-9-aminoacridines, which were synthesized to achieve double intercalating compounds, also showed increased cytotoxic and antitumor activity. However, a lack of correlation between antitumor efficacy and cytotoxicity in cell cultures was found, and subsequently it was discovered that the antitumor activity did not correlate at all with DNA and RNA synthesis inhibition, or with rate of drug uptake in cell culture, but correlated with the effect on the rate of lectin-induced cell agglutination, a cell-surface phenomenon. This experience demonstrates that compounds endowed with good affinity for DNA can also exert their antitumor effect through other mechanisms.

In the field of anthracyclines, preliminary observations (10) suggested that alteration of cell membranes could be an important effect. Of fundamental importance were the observations reported by Tritton and Yee (11) and by Tokes et al (12), about the cytotoxic activity in vitro of doxorubicin bound to macromolecules, in conditions which completely prevented the entrance of the drug into the cell and therefore any possible interaction with nucleic acids. More recent studies have shown that, following photoirradiation of daunorubicin-treated cells, the drug can be found bound to proteins, not to DNA or lipids (13). Binding probably occurs at the cell surface, and can be at cell-surface proteins which, although not directly involved in the mechanism of cytotoxicity, are implicated

[1]Biological Research and Development, Farmitalia Carlo Erba Research Center, Milan, Italy.

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[3]While this review was being prepared, a new anthracycline, menogaril, has been introduced into clinical trials, according to different schedules: Zimet AS, Ohnuma T, Perlow L, et al, A phase I trial of menogaril (7-CON-*O*-methylnogarol) in a daily × 3 schedule, Proc Am Assoc Cancer Res 26:167, 1985; Sigman LM, Van Echo DA, Egorin MJ, et al, Menogaril (7-OMEN, NSC 269148) phase I study, Proc Am Assoc Cancer Res 26:170, 1985; Brown TD, Donehower RC, Grochow LB, et al, A phase I study of menogaril (MEN) in patients with advanced cancer, Proc ASCO 4:32, 1985; Dorr A, Von Hoff D, Kohn J, et al, Phase I clinical trial of menogaril, Proc ASCO 4:39, 1985; Long HJ, Powis G, Schutt AJ, et al, Menogaril by 72-hour continuous intravenous infusion: a phase I study with pharmacokinetics, Proc ASCO 4:28, 1985; and Dodion P, Sessa C, Gerard P, et al, Phase I trial of menogaril (M) with a single dose schedule, Proc ASCO 4:26, 1985.

[*]*Reprint requests to:* Dr. A. M. Casazza, Pharmaceutical Research and Development Division, Bristol-Myers Co, PO Box 4755, Syracuse, NY 13221-4755.

TABLE 1.—Suggested biochemical mechanisms of anthracyclines

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|---|
| Binding to DNA |
| Inhibition of DNA and RNA polymerases |
| Induction of DNA breaks |
| Free-radicals formation |
| Cell membrane alteration |
| Mitochondrial alteration |
| Inhibition of ion exchange |
| Binding to calmodulin |
| Binding to phospholipids |
| Binding to cardiolipin |

in the expression of drug resistance. Unfortunately, no new anthracycline has been found up to now, which clearly shows antitumor activity disassociated from the ability of binding to DNA. AD-32 has been described as a new analog endowed with antitumor activity at least equal to that of doxorubicin (14). This compound does not exhibit any ability of binding to DNA in cell-free systems (15). Utilizing radioactive AD-32 labeled at position 14, Zini et al (16) have recently been able to show that AD-32, administered iv to rats, is converted to *N*-trifluoroacetyldoxorubicin, 13-dihydro-*N*-trifluoroacetyldoxorubicin, and doxorubicin. Doxorubicin was the main urine metabolite, and was also present in relevant amounts in the tissue extracts examined: liver, kidney, spleen, and lung. Even if not completely explanatory of the mechanism of action of AD-32, these data strongly suggest that conversion to a DNA-binding anthracycline species can be relevant to explain the biologic activity of AD-32. Also, these data support the importance of metabolism studies with anthracyclines, a field which only recently has received attention.

A new class of anthracyclines has recently been described; it is constituted by 3′-morpholinyl and 3′-CN-morpholinyl anthracyclines (17,18). These compounds are of great interest because of the extremely high cytotoxicity, higher potency than the parent drugs, and activity against doxorubicin-resistant tumors (19). In an attempt to explain the reasons for these features, the mechanism of action has been investigated. At variance with doxorubicin was the higher inhibition of RNA than of DNA synthesis in cell cultures (20). Other anthracyclines have been described in the past which preferentially inhibit RNA synthesis (21), such as aclarubicin and marcellomycin. They have been grouped into the Class II anthracyclines, in contrast to Class I anthracyclines, which show similar inhibition of DNA and RNA synthesis and include doxorubicin. After the description of morpholinylanthracyclines, Class II anthracyclines include compounds which are less cytotoxic than doxorubicin in vitro and less potent in vivo, such as aclarubicin, as well as the extremely potent morpholinyl derivatives. These data show that there is no correlation between ability to preferentially inhibit RNA synthesis and potency in exerting biological effects.

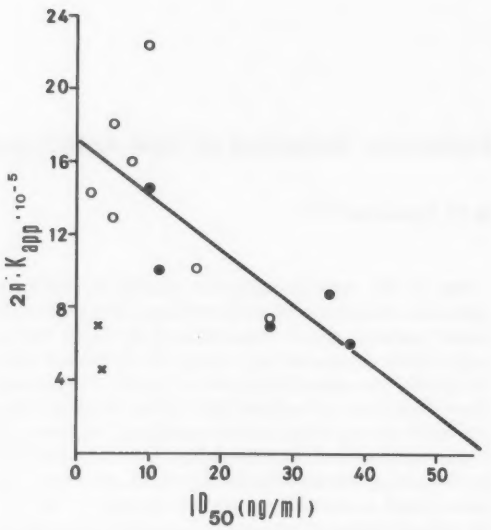


FIGURE 1.—Relationship between cytotoxicity and ability to bind to DNA of anthracycline derivatives. *Abscissa*—ID50 on HeLa cells (ng/ml); *ordinate*—$2n \cdot K_{app} \cdot 10^{-5}$. ● = daunorubicin and its derivatives; ○ = doxorubicin and its derivatives; X = 4′-epi-4′-C-methyldaunorubicin and 4′-epi-4′-C-methyl-4′-*O*-methyldaunorubicin, which were not considered for the calculations of the linear regression. Correlation coefficient was = -0.76; intercept = 17.1; slope = 0.302 (ref 7).

The inhibition of nucleic acid synthesis by CN-morpholinyl doxorubicin seems to be the consequence of an interaction with DNA that is different from the intercalative binding of classical anthracyclines. Studies by Johnston et al (18) suggested either an additional nonintercalative mode of binding or an alternated base-pair specificity by this drug. The possibility of the formation of an adduct between CN-morpholinyl doxorubicin and a base in DNA was also advanced. Actually, it has been subsequently demonstrated that some cyanoporpholino derivatives exhibit an irreversible binding to DNA (22). For this property, morpholinyl anthracyclines should be considered as a new class of antitumor agents, which deserve deep and careful investigation.

## Biologic Activity

Because the mechanism of action of anthracyclines has not yet been established, it has not been possible up to now to identify one or more biochemical tests which could allow the rapid selection of new analogs. Therefore, evaluation must be done by adopting in vitro tests in cell cultures or in vivo tests.

### *Antitumor Activity*

In previous reviews, the relevance of some experi-

mental models has been discussed as far as predictivity of clinical effectiveness is concerned (23,24). Experimental leukemias seem to be good models of human leukemia. In fact, compounds which showed activity at least equal to doxorubicin or daunorubicin in mouse leukemias proved to be active antileukemic agents in patients: eg, zorubicin (25) and epirubicin (26,27). Idarubicin, which was more active than daunorubicin and doxorubicin against some mouse leukemias (28), has an impressive antileukemic activity in patients, even when heavily pretreated (29), confirming a good correlation between experimental and clinical data.

The picture given by solid tumors is much more complicated. There are some good examples of positive correlation. The mammary carcinoma of C3H mice predicted well the antitumor activity of doxorubicin, and later of epirubicin, against breast cancer in women (30). In a comparative clinical trial, epirubicin was effective in this particular type of tumor, while carminomycin, which has a lower activity than doxorubicin against experimental tumors, such as L1210 leukemia, B16 melanoma, and Lewis lung carcinoma (31), was ineffective (32). On the other hand, zorubicin gave variable results against some mouse solid tumors, such as sc B16 melanoma and Lewis lung carcinoma (31), and was less active than doxorubicin in patients with solid tumors (33).

These data suggest that, working with a rather homogeneous class of compounds, such as anthracyclines, some transplanted mouse tumors can be identified which can be considered predictive of the clinical data. Surely, this is not the case if working on unknown chemical structures, where predictivity has been so far rather low so that there is a real need for setting up new experimental models (34). In this regard, it can be interesting to examine the data obtained with anthracyclines tested against human tumors in "extra-clinical" trials, such as human tumor stem cell assay (HTSCA) and heterotransplantation of human tumors into nude mice.

In an important analysis carried out on tumors in 4916 patients (35), doxorubicin activity in HTSCA correlated well with response rates in patients as reported in the literature. The data encouraged further studies of new anthracyclines in this model (36-38). In one study, the activity found in these systems was similar to that observed using HeLa cells (36). In other studies, some analogs showed activity higher than that of doxorubicin either against a specific tumor type, eg, epirubicin in ovarian carcinomas (39,40), or against various tumors, eg, esorubicin (37).

What is the correlation between data obtained in HTSCA and those in nude mice? A direct comparison has been carried out by Teatle et al (38). In vivo tumor growth delay and maximal in vitro cytotoxicity for epirubicin and doxorubicin showed a significant correlation. Moreover, separation of tumors into "sensitive" and "insensitive" tumor population on the basis of in vitro effect predicted the in vivo response to these two drugs in 88% of cases.

Contrary to these encouraging results are the rather puzzling data obtained with esorubicin. This anthracycline showed higher activity than doxorubicin against several in vivo models of colon carcinomas such as human tumors transplanted in nude mice (41), mouse transplanted adenocarcinomas (42), and carcinogen-induced mouse colon tumors (43). On the other hand, these results were not paralleled by the in vitro data. In fact, esorubicin showed interesting activity in HTSCA against melanomas, leukemias (44), ovarian cancer (36), and other human malignancies (37), but gave conflicting results on human colon tumors. No activity was found by Salmon and Durie (44) against colon tumors, whereas moderate activity which, however, did not parallel in vivo data in nude mice, was reported by Teatle et al (38).

In a second study on these same tumors, esorubicin showed activity in vitro against four of them, and the sensitivity ranking deduced from the in vitro experiments paralleled, in this case, the in vivo data (45). In this study, an interesting observation was made: esorubicin was active against growing populations of primary cultures of T 348 human colon carcinoma, but no effect was found on stationary populations of the same cells. One of the puzzling questions in the anthracycline field has always been the reason for the higher effectiveness of doxorubicin over daunorubicin against solid tumors (46); the most likely hypotheses made during the years have been: (*a*) different immunomodulating properties (47,48); (*b*) more favorable pharmacokinetic properties (49); and (*c*) ability to inhibit all proliferating cells, no matter where they are in the cycle at the time of treatment, and probably also resting-phase cells, which are in general rather resistant to chemotherapy (50). Disease-oriented phase II trials in solid tumors, and specifically in colorectal carcinoma, of esorubicin are of particular importance in light of the above-reported experimental observations. If esorubicin will not be found more active than doxorubicin on colon cancer, hypothesis (*c*) will be strongly enforced, and the whole problem of in vitro tests for evaluation of antitumor agents will have to be reconsidered.

#### *General Toxicity*

Ten anthracyclines have been examined with regard to the relationship between experimental toxicity, evaluated through the LD50 in mice treated iv, and the maximal tolerated dose or the optimal dose for patients treated every 3 weeks iv (24).

A summary of these observations is shown in table 2. It can be observed that, with the exceptions of marcellomycin and 4′-*O*-tetrahydropyranyl-doxorubicin, the anthracyclines that were less toxic than doxorubicin in mice were also less toxic in patients (eg, epirubicin, aclarubicin, and zorubicin), and those that were more toxic than doxorubicin in mice (eg, carubicin and idarubicin) con-

TABLE 2.—Correlation between experimental and clinical toxicity of anthracyclines

| Toxicity and drug | Mice (mg/kg iv)* | Clinical dose ($mg/m^2$ iv every 3 wks) |
|---|---|---|
| Doxorubicin | 10-13 | 60-75 |
| Less toxic in mice and patients | | |
| Daunorubicin | 18 | > 70 |
| Epirubicin | 16-19 | 75-90 |
| Aclarubicin | 32 | 133 |
| More toxic in mice and patients | | |
| Carminomycin | 3.7 | 20 |
| Idarubicin | 4.8 | 15-18 |
| Less toxic in mice, more toxic in patients | | |
| Marcellomycin | 19.9 | 50 |
| THP-doxorubicin | 28 | 50 |

*LD50.

firmed such finding in man. In this field, therefore, a good correlation between preclinical and clinical data has been found, despite the difference in metabolism, which will be discussed later.

### *Cardiac Toxicity*

Cardiac toxicity is known as one of the limiting factors in the clinical application of anthracyclines, and several studies have been devoted to a better understanding of it, from a clinical as well as from a basic point of view. It has been possible to set up experimental models (51) which have been shown to be predictive of the clinical data, as recently discussed (30,52). The best example of positive correlation between experimental and clinical data is given by epirubicin. This compound was 25% less cardiotoxic than doxorubicin in animals (28). In man, not only was epirubicin confirmed to be less cardiotoxic than the parent drug, but the magnitude of difference was amplified to about 50% (53). Similarly, animal tests correctly predicted the low cardiotoxicity of aclarubicin and esorubicin. Pharmacokinetic data have been provided which can explain these differences (54).

What still is missing is a better understanding of the pathogenesis of the myocardial injury. In particular, one important question has not yet been answered. "Is the cardiac toxicity due to the same biochemical mechanisms which sustain antitumor activity?" The availability of anthracyclines in which cardiac toxicity and antitumor activity are dissociated, such as esorubicin (52), would suggest a negative answer to this question. However, too many differences in pharmacokinetics and metabolism exist between the anthracyclines investigated so far (discussed later) to allow definite conclusions. On the other hand, many biochemical mechanisms have been evoked in relation to anthracycline cardiotoxicity; some of these differ from those which are supposed to be at the base of the cytotoxic activity. Compounds like epirubicin and esorubicin, which are less cardiotoxic than the parent drug, have been shown to concentrate less than doxorubicin in the heart (55) *and* to inhibit less than doxorubicin the $Na^+/Ca^{++}$ exchange in isolated dog heart sarcolemmal vescicles (56).

The low cardiotoxicity of epirubicin and esorubicin might conceivably be related to the lower effect on $Ca^{++}$ exchange at the heart level, together with the favorable pharmacokinetic properties mentioned above. Preliminary studies have shown that esorubicin was as active as doxorubicin on DNA and RNA synthesis in mouse hearts (57), and was unable to generate oxygen-containing free radicals, as measured by electron-spin resonance spectra of aqueous solutions in the presence of the spin trap, phenyl-*N*-t-butylnitrone at pH 7.5 (58). These studies need confirmation, but are suggestive of a mechanism of cardiac toxicity which can be related with the ability to generate free radicals, and do not seem to depend on inhibition of nucleic acids.

## Resistance

The importance of finding new anthracyclines active against anthracycline-resistant cells is clinically relevant. Unfortunately, this important requirement has not been followed by the appropriate experimental work, and a primary screening of new anthracycline analogs against resistant tumor cells is not routine. All of the compounds which entered clinical trials have, however, been tested against doxorubicin-resistant P388 leukemia (P388/doxorubicin), which represents the standard model for resistance studies (59); none of them showed substantial activity.

To increase the capability to evaluate new anthracyclines on resistant cells, we have recently set up a method in vitro which allows the contemporary evaluation of cytotoxicity on P388 and P388/doxorubicin (60). A certain correlation seems to be present between the in vitro and in vivo data on P388/doxorubicin. In particular, in this study we have selected CN-morpholinyl doxorubicin, 4-demethoxy-4′-*O*-methyldaunorubicin (61), and, more recently, the class of 4′-halo anthracyclines (62), and studies have been performed to understand the mechanisms by which these compounds exert their cytotoxic activity against the P388 leukemia line with acquired resistance to doxorubicin (P388/doxorubicin). It has been previously reported that, in most cases of acquired experimental resistance to anthracyclines, there is a decreased accumulation of the drugs in the resistant cells compared to the wild-type cells (63). The uptake and efflux of these drugs into P388/doxorubicin cells has therefore been measured in comparison with the P388-sensitive cells in vitro. The results supported the hypothesis that membrane permeability to anthracyclines is one of the factors by which resistant tumor cells, through a reduction of drug accumulation, express their drug resist-

ance (61,64), but suggested that other factors, besides drug accumulation, can be involved. It has been pointed out (63) that the decreased drug accumulation observed in many anthracycline-resistant cells is not necessarily the only reason for the resistance. In Ehrlich ascites tumor cells, it was observed that the same intracellular daunorubicin concentration caused considerably less inhibition of nucleic acid synthesis in the resistant than in the wild-type cells (65). Since the problem of cell resistance is of great clinical relevance, it is of primary importance at this point to clarify whether anthracycline resistance is due to altered cell transport. It is still not known whether the mechanisms of resistance to anthracyclines of P388 leukemia cells also operate in vivo; further studies in this field are needed. It has been shown in human ovarian cancer cell lines that verapamil, a calcium channel-blocking agent, is able to circumvent doxorubicin resistance (66). The potentiating effect of verapamil was due to inhibition of doxorubicin efflux in the resistant cells. This study is of particular importance, since it shows that (*a*) resistant human cancer cells exhibit biochemical mechanisms similar to those observed in mouse cancer cells; and (*b*) in human tumors anthracycline resistance can be due to decreased intracellular drug accumulation.

Development of these studies, together with increased comprehension of the pharmacokinetic properties of anthracyclines at the cellular and intracellular level, will hopefully give us a more rational approach to the problem of cell resistance.

### Pharmacokinetics and Metabolism

The importance of these studies in the field of antitumor agents does not need to be stressed. With regard to anthracyclines, no general correlations between chemical structure and pharmacokinetic properties have been established, except the dependence of intracellular drug concentration on lipid solubility (as tested by partition coefficients in *N*-butanol aqueous buffer) suggested for groups of homogeneous derivatives (23).

However, several studies in recent years have contributed to our knowledge on this important class of antitumor agents. It was suggested that the more favorable therapeutic properties of epirubicin in comparison with doxorubicin in mice could be due to the different tissue levels of the parent drug in target organs, such as spleen and heart (55). In addition, studies with unlabeled or $^{14}C$-labeled epirubicin in mice (67) and rats (68) have shown that the main metabolite was 13-hydroxyderivative, suggesting a metabolic pattern qualitatively similar to that of the parent drug. However, in man it was found that the metabolism of epirubicin differs from that of doxorubicin, since 4′-*O*-β-D-glucuronides of both the parent compound epirubicin and 13-hydroxymetabolite are formed, at variance with doxorubicin, which is glucuronized only after hydroxylation at position 13 (69,70). Only traces of these metabolites were found in rats. The important finding of the different metabolization of epirubicin in animals and in patients can explain the further reduction of cardiotoxicity when going from experimental systems to the clinic. The 13-OH metabolites of both epirubicin and doxorubicin are less active than the parent drug (71). On the contrary, 13-OH-carminomycin and 13-OH-idarubicin were shown to be as active or more active than the respective parent drugs (72,71). Since these metabolites rapidly reach plasma levels higher than the parent drugs, the possibility also exists with these analogs that biologic properties can be different in animals and man.

The two examples described above show that pharmacokinetics and metabolism need to be extensively investigated in preclinical models and in the clinic, since the experimental data are not always predictive of the clinical ones. The possibility to set up preclinical models which can allow the study of anthracycline metabolism by human cells in vitro should be intensively pursued.

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# Clinical Assessment of the Structure-Activity Relationship of Anthracyclines and Related Synthetic Derivatives

**Charles W. Young* and Virginia Raymond[1,2]**

The anthracyclines are of primary importance in cancer treatment and present a fascinating challenge to the medicinal chemist, pharmacologist, chemotherapist, and oncologist. They produce multiple differing biologic effects, including major therapeutic activity in patients with either acute leukemia or solid tumors. Unfortunately, they damage proliferating noncancerous tissues as well, primarily the bone marrow and hair follicles. They also produce intense local tissue injury when extravasated, and most significantly, acute and chronic cardiotoxicity. In seeking new anthracyclines, the goals of drug development are as follows: to identify more selective molecules; to separate the multiple biologic activities that are manifested by the currently used anthracyclines, doxorubicin and daunorubicin, and to retain and if possible increase the desirable cytotoxic effects against cancer cells and reduce the undesirable effects on the heart and non-neoplastic proliferating tissues.

Preclinical scientists use a variety of model systems to evaluate newly discovered or synthesized anthracyclines for possible entry into clinical trial. These include a spectrum of murine and human tumor cell lines, with varying degrees of responsiveness to anthracyclines, growing as leukemias or solid tumors and as xenografts in immunodeficient "nude" mice. There are model systems also for the drugs' cardiotoxic effects, including both acute (largely in vitro) models and chronic toxicity models in several animal species.

A number of new anthracycline analogs have been taken into clinical evaluation because of their apparent increased selectivity in these models. For the most part, these compounds retained anticancer effects with reduction of cardiotoxicity. The results of the clinical trials provide not only an evaluation of the drugs under study but also an assessment of the concept that had led to their genesis and the predictability of the model system in regard to clinical results. This review will include not only consideration of the classic anthracyclines but also discussion of the purely synthetic compound mitoxantrone, which was developed based on a conceptual hypothesis regarding the mechanisms of the anthracylines' anticancer and cardiotoxic effects.

It is impossible, in the framework of this review article, to discuss all of the compounds that have reached clinical trial, or to list all of the articles on those that are discussed extensively. In our selection of topics and references, we have made an effort to emphasize those compounds where there is an abundance of clinical data, and particularly those where investigation is ongoing and wide eventual usage of the compound appears likely. The articles selected for listing provide key conceptual information or a point of entry into the relevant literature.

## STRUCTURAL CONSIDERATIONS

This review will begin with a consideration of molecules which present structural modifications of a rather simple nature on doxorubicin and daunorubicin and then progress to those where the structural changes are of greater complexity. It will begin with compounds that have alterations on the aminosugar, daunosamine, then to drugs with changes on the chromophore, and thereafter to the more complex alterations where changes are made on both structures.

Before beginning the detailed review, certain points can be made of general relevance. The acute dose-limiting toxic effect of all of the anthracyclines is myelosuppression, with the severity of leukopenia exceeding that of thrombocytopenia. Nausea and vomiting may be decreased with some analogs, but they are rarely absent. In contrast, the severity of alopecia may vary considerably among the different compounds. Chemically, the majority of the newer analogs are more lipophilic than doxorubicin and enter into and efflux from cells more readily in model systems. In this, their characteristics resemble those of daunorubicin rather than doxorubicin, and like daunorubicin, they commonly show an ability to induce remission of acute leukemia. Some of them are also like daunorubicin in that they do not have major activity against solid tumors.

[1]Developmental Chemotherapy Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, Cornell University Medical College, New York, NY.

[2]We thank Ms. Maryann Herman and Ms. Wendy Werner for preparation of the manuscript.

*Reprint requests to: Dr. Charles W. Young, Developmental Chemotherapy Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

## ANALOGS OF DOXORUBICIN

### Epirubicin

Epirubicin, differing from doxorubicin only through epimerization of the 4′ hydroxyl on the daunosamine, presents perhaps the least structural departure of the new analogs that have received clinical trial (1). Although the biological effects of the resultant molecule are similar to those of doxorubicin, there are sufficient differences to make the new analog clinically interesting. It has an antitumor spectrum in mice that is similar to that of doxorubicin, but it produces less host toxicity, including less cardiotoxicity (2-4).

Through extensive clinical trials it has been established that epirubicin has therapeutic activity in the tumor varieties that are classically responsive to doxorubicin. It is also clear that epirubicin is less myelosuppressive than doxorubicin when the two drugs are given at equal dosage and less cardiotoxic than doxorubicin whether given at equal dosage or equally myelosuppressive dosage. The principal issue presently being addressed is whether epirubicin and doxorubicin produce equal therapeutic effects when they are given at equal dosage, or whether larger doses of epirubicin are required to produce therapeutically equivalent results. If and where the former is true, epirubicin would be a more selective anthracycline; in the latter circumstance, it would merely be a less potent anthracycline.

Epirubicin and doxorubicin produce quite similar effects on tumor cell lines in culture; they are comparably cytotoxic, inhibit synthesis of DNA and RNA, and produce strand breakage of DNA. In most cytotoxic systems the two drugs are of comparable potency. In contrast, clear differences exist between the effects of the two drugs on myocardial cells and myocardial sarcolemmal vesicles. Both doxorubicin and epirubicin inhibit calcium exchange and decrease myocardial contractility in guinea pig atria through inhibition of the sarcolemmal membrane $Na^{++}/Ca^{++}$ exchanger; however, epirubicin is less than half as potent an inhibitor of both processes (5,6).

#### *Clinical Pharmacology and Metabolism*

The clearance of both epirubicin and doxorubicin from plasma is triexponential, with a rapid distribution phase and a prolonged elimination phase consistent with extensive drug retention within peripheral tissues and gradual release thereafter. The overall pharmacokinetic patterns of epirubicin and doxorubicin and their 13-OH metabolites are similar; however, the clearance of epirubicin is faster, whether examined on comparison of treated populations or on a cross-over basis in patients given both drugs (7-9). In man, the metabolism of the two compounds differs in that there is extensive formation and circulation of the 4′-β-D-glucuronide conjugate of both epirubicin and epirubicinol; commonly the combined area under the curve (AUC) of the glucuronide conjugates exceeds that of epirubicin itself (7-10). In their detailed studies of the urinary metabolites of doxorubicin and daunorubicin, Takanashi and Bachur (11) identified glucuronides of the aglycones; however, glucuronide conjugation with the daunosamine moiety was not observed.

Although the above-described rapid clearance of epirubicin, and extensive circulation of glucuronide metabolites thereof, could account for an observed difference in man in the overall toxicity between that drug and doxorubicin, it offers no assurance of preservation, in man, of the drug's anticancer potency. However, although glucuronide metabolites are widely considered to have minimal entry into somatic cells, this is not universally true in regard to anticancer drugs. Animal tumors with high β-glucuronidase content have the capacity to activate in situ circulating glucuronides of active compounds, such as *p*-OH-aniline mustard, with a resultant major antitumor effect (12).

#### *Toxic and Therapeutic Effects*

Epirubicin has received extensive study in a broad variety of tumor types; for a detailed statement of the phase I trials or the early clinical phase II trials, one may refer to the extensive review by Ganzina (13). The most extensively used regimens employ intermittent dosage at 21-day intervals. Given in this manner, the dose-limiting toxic effect is myelosuppression, with the severity of leukopenia exceeding that of thrombocytopenia. Epirubicin (85-90 $mg/m^2$) was judged to be comparably myelosuppressive to 60 $mg/m^2$ of doxorubicin. Epirubicin also produces nausea, vomiting, alopecia, local necrosis when extravasated, and cardiac injury on long-term usage. The relative severity of these side effects in comparison to doxorubicin is addressed in the context of prospectively randomized comparison studies that are described below. Epirubicin has therapeutic effects in most of those tumor varieties that are sensitive to doxorubicin. The results of multiple studies are summarized in table 1 (14).

#### *Studies in Breast Cancer*

The comparability of epirubicin's activity in breast cancer to that of other anthracyclines has been addressed in a series of prospectively randomized single-agent trials versus carminomycin or doxorubicin and in a prospectively randomized comparison of the multidrug regimens FEC and FAC, ie, epirubicin or doxorubicin in otherwise identical regimens of 5-FU and cyclophosphamide. The results to date of these trials are presented in table 2 (15-20).

Epirubicin was distinctly superior to carminomycin (15) and produced comparable complete response plus partial response (CR + PR) rates to those of the respective doxorubicin-containing arms (16-20). The study

TABLE 1.—Summary of phase II results of epirubicin

| Tumor type | No. of evaluable patients | No. of CRs + PRs | % of CRs + PRs |
|---|---|---|---|
| Breast carcinoma | 237 | 57 | 24 |
| Breast, no prior chemotherapy | 55 | 20 | 36 |
| Breast, prior chemotherapy | 182 | 37 | 20 |
| Soft tissue sarcoma | 133 | 19 | 14 |
| Ovarian carcinoma | 41 | 15 | 36.5 |
| Non-Hodgkin's lymphoma | 32 | 23 | 72 |
| Colorectal carcinoma | 283 | 23 | 8 |
| Rectum, rectosigmoid | 121 | 16 | 13 |
| Colon carcinoma | 162 | 7 | 4 |
| Gastric carcinoma | 76 | 13 | 17 |
| Pancreas carcinoma | 50 | 17 | 34 |
| Hepatocellular carcinoma | 38 | 10 | 26 |
| Lung carcinoma | | | |
| Non-small cell | 218 | 13 | 6 |
| Small cell | 13 | 5 | 38 |
| Malignant melanoma | 161 | 10 | 6 |
| Renal carcinoma | 75 | 4 | 5 |
| Bladder carcinoma | 15 | 3 | 20 |

arms were quite comparable also in remission duration and the time to progression of the treatment populations. When the two drugs were given in equal dosage, the patients receiving epirubicin experienced less myelosuppression than those receiving doxorubicin. As discussed below, these studies also demonstrated epirubicin to be significantly less cardiotoxic than doxorubicin.

Although these results certainly confirm the activity of epirubicin, they do not conclusively establish its comparability at equal dosage; the Memorial Sloan-Kettering Cancer Center (MSKCC) and the European Organization for Research on Treatment of Cancer (EORTC) studies used unequal dosages of the two drugs, while the National Cancer Institute (NCI)-Milan study (which employed equal dosages) contained only 21 patients in each arm. The FAC versus FEC study has enlisted sufficient patient numbers to address the comparable potency issue; however, the presence of the two other drugs in the regimen confounds the interpretation of results. This issue is being examined in further single-agent and combined-regimen studies presently in process.

### *Studies in Soft Tissue Sarcoma*

In contrast to the circumstances with breast cancer, the observed CR plus PR rates to date in the EORTC sarcoma comparison study, which uses equal doses of doxorubicin and epirubicin, are 24% (18 of 75 patients) and 15% (11 of 75), respectively (21). Although this difference is not significant ($P = 0.21$), when the test for a linear trend was applied, the response data were in favor of doxorubicin. In spite of this trend, the time to progression and the survival curves of the two groups overlap. At the dosages given, 75 mg/m$^2$ for each drug, there was a significant difference in the observed toxicity between the two treatment groups. The initial course leukocyte count nadirs were lower in the doxorubicin-treated patients ($P = 0.007$); nausea and vomiting and alopecia (requiring a wig) were also more severe in the doxorubicin arm, with $P = 0.05$ and $P = 0.0008$, respectively. A follow-up study in soft tissue sarcomas employing equimyelosuppressive regimens is planned.

### *Studies in Other Tumor Varieties*

Epirubicin also has meaningful activity in ovarian cancer (table 1) and malignant lymphoma (14). In these disorders epirubicin is also being examined as a component of combination therapy (22); however, a detailed comparative assessment with doxorubicin or a doxorubicin-containing regimen has not yet been published. The drug's activity in gastrointestinal cancer requires further definition. The drug does not have significant therapeutic activity in adenocarcinoma of the colon (table 1). However, Falkson and Vorobiof (23) have reported a 16% CR plus PR rate in rectosigmoid and rectal cancer, with 13 responses among 52 previously untreated patients.

Doxorubicin has at least marginal activity against both gastric and pancreatic cancer, sufficient to produce objective regression of disease and make the FAM regimen widely used in the treatment of those disorders. Similarly, epirubicin has activity of potential interest in carcinoma of the stomach, pancreas, and liver (24-26). The reported trials as yet still involve relatively small numbers of patients; extension of the work is being actively pursued.

Epirubicin does not have major activity against non-small cell lung cancer but has produced occasional responses in head and neck cancer. In the latter disorder combined regimens of cisplatin and epirubicin are being

TABLE 2.—Prospectively randomized comparison of anthracyclines in breast cancer and soft tissue sarcoma

| Study population | Institution or group | Regimen * | No. of evaluable patients | No. of patients with CR + PR (%) |
|---|---|---|---|---|
| Breast cancer, previously treated, anthracycline-naive | EORTC: early clinical trials group | Epirubicin (90 mg/m$^2$) | 34 | 9 (26) |
| | | vs | | |
| | | Carubicin (18 mg/m$^2$) | 29 | 1 (3) |
| Breast cancer, previously treated, anthracycline-naive | MSKCC | Epirubicin (85 mg/m$^2$) | 24 | 6 (25) |
| | | vs | | |
| | | Doxorubicin (60 mg/m$^2$) | 28 | 7 (25) |
| Breast cancer, previously untreated and previously treated, anthracycline-naive | NCI-Milan | Epirubicin (75 mg/m$^2$) | 21 | 13 (62) |
| | | vs | | |
| | | Doxorubicin (75 mg/m$^2$) | 21 | 11 (52) |
| Breast cancer, prior chemotherapy, anthracycline-naive | EORTC: early clinical trials group | Epirubicin (90 mg/m$^2$) | 31 | 10 (32) |
| | | vs | | |
| | | Doxorubicin (75 mg/m$^2$) | 33 | 10 (30) |
| Breast cancer, previously untreated and postadjuvant relapse, anthracycline-naive | French epirubicin study group | FEC | 112 | 52 (46.5) |
| | | vs | | |
| | | FAC† | 105 | 52 (49.5) |
| Soft tissue sarcoma, no prior chemotherapy | EORTC | Carubicin (20 mg/m$^2$) | 33 | 1 (3) |
| | | vs | | |
| | | Doxorubicin (75 mg/m$^2$) | 38 | 11 (29) |
| Soft tissue sarcoma, no prior chemotherapy | EORTC | Epirubicin (75 mg/m$^2$) | 75 | 11 (15) |
| | | vs | | |
| | | Doxorubicin (75 mg/m$^2$) | 75 | 18 (24) |

* Every 21 days.
†Epirubicin (50 mg/m$^2$) vs doxorubicin (50 mg/m$^2$), both with cyclophosphamide (500 mg/m$^2$) and 5-FU (500 mg/m$^2$).

examined. The drug has been studied extensively in patients with malignant melanoma with essentially negative results.

### *Cardiotoxicity*

The prospective comparative trials in breast cancer, an anthracycline-responsive tumor, have provided detailed information on the two drugs' relative cardiotoxic effects. The MSKCC trials did not use a preselected maximal cumulative dose for either drug; rather, it called for drug continuance in each patient until there was tumor progression or laboratory or clinical evidence of significant myocardial dysfunction. Laboratory assessment of cardiac function consisted of resting and exercise radionuclide cineangiography with Fourier amplitude and phase analysis, carried out prior to the first course of therapy and after Cycles 3, 5, 7, 9, etc. Laboratory cardiotoxicity was defined as a $> 10\%$ drop in the resting left ventricular ejection fraction (LVEF) from the baseline value or a $> 5\%$ decrease in LVEF on exercise below the resting LVEF obtained on that day.

Nine patients receiving doxorubicin had laboratory cardiotoxicity with a median cumulative dose of 350 mg/m$^2$, contrasting with a median cardiotoxic dose of 892 mg/m$^2$ for epirubicin in seven patients (16). Six patients receiving doxorubicin experienced heart failure, with a median dose of 562 mg/m$^2$; four patients receiving epirubicin experienced heart failure, with a median dose of 1134 mg/m$^2$. The differences were highly statistically significant and independent of known pre-existing risk factors for anthracycline cardiotoxicity. The favorable cardiotoxic experience with this epirubicin-treated breast cancer population is consistent with other observations in anthracycline-naive solid tumor patients, where the appearance of heart failure has been observed only at dosages $> 1000$ mg/m$^2$.

The cardiotoxic potency relationship between doxorubicin and epirubicin in the above study was 2 to 1. The endomyocardial biopsy data of Torti et al (27) on a more heterogeneous population has yielded an estimated potency relationship of the two drugs of 1.8 to 1. In the NCI-Milan comparison and the French FAC versus FEC studies cumulative dose limitations were employed for both drugs; however, two patients on the doxorubicin arm in the former study and three patients on the FAC arm in the latter developed heart failure. There were no episodes of heart failure on the epirubicin study arms.

## Esorubicin

Esorubicin differs from doxorubicin by being dehydroxylated at position 4′ on the aminosugar (28). This modification results in increased basicity of the amino

group and increased lipophilicity (2-4). Esorubicin is more potent than doxorubicin, enters cells more rapidly, and is cleared more rapidly from plasma (2-4,29,30). It was advanced to clinical trial because of its comparability to doxorubicin in most model tumor systems, its superiority in human tumor xenografts in nude mice (31-33), and its greatly reduced or absent cardiotoxicity in animals (3,4). In clinical assessment to date it has proven to be clearly inferior to doxorubicin in breast cancer and inactive in colorectal and non-small cell lung cancers; assessment in acute leukemia and in other solid tumor varieties is ongoing.

Esorubicin attracted considerable research interest because of its apparent superiority to doxorubicin and to other anthracycline analogs against human colorectal tumor cell lines growing as xenografts in nude mice (31-33). It was noted by Taetle et al (33) that esorubicin was less effective in clonogenic reduction in the human tumor cell lines on in vitro exposure than against the same lines growing as xenografts in vivo; accordingly, the clinical assessment of esorubicin was looked upon as a means of examining the clinical predictability of studies in human cell lines. Although the positive results in the xenograft system have not been supported by the clinical findings (*see* below), the reasons for the discrepancy are not as yet clear.

In both mice and man, esorubicin is cleared from plasma in a triphasic fashion, with a very rapid initial phase and a prolonged terminal phase (30,34). In mice, the principal circulating metabolites are the aglycones of esorubicinol; in man, esorubicinol is the predominant metabolite detected.

Phase I assessment established that myelosuppression was the dose-limiting toxic effect of esorubicin; significant alopecia was rare except at high dosage, and nausea and vomiting was mild. The available clinical data do not permit a meaningful comment on the drug's cardiotoxic potential. The recommended phase II dosage was 30-35 mg/m$^2$ at 21-day intervals.

Although detailed clinical assessment of the drug is still ongoing, the available data indicate that it does not have significant activity against colorectal carcinoma. When the EORTC and the MSKCC data were combined, esorubicin therapy produced no major responses among 97 evaluable patients, 40 of whom had not received prior chemotherapy (35; Kemeny NK, unpublished data). Esorubicin is also inferior to doxorubicin and epirubicin in breast cancer patients, producing major responses in five of 24 patients and in none of 20 patients in the two series reported to date (36,37). One negative report in non-small cell lung cancer has appeared; the drug has not shown useful activity in sarcomas (Magill GB, unpublished data).

### 4'-O-Tetrahydropyranyladriamycin (THP-ADM)

THP-ADM was synthesized by Umezawa et al (38), Tsuruo et al (39), and Dantchev et al (40) and advanced to clinical trials because of its favorable behavior in transplanted leukemias and solid tumors and its reduced cardiac toxicity. Like many of the analogs discussed previously, it has greater lipophilicity than doxorubicin, more rapid entry into cells, and more rapid clearance from plasma (41); it appears to be catabolized in a fashion similar to that of doxorubicin, with formation of the 13-OH derivative and subsequent formation of the aglycone. In the initial clinical trials in Japan, myelosuppression has been the dose-limiting toxic effect and alopecia was mild; therapeutic responses have been seen in patients with gynecologic cancer and non-Hodgkin's lymphoma (42). These clinical trials will undoubtedly be expanded.

## ANALOGS OF DAUNORUBICIN

### Idarubicin

Idarubicin differs from its parent compound, daunorubicin, in the replacement of the C-4 methoxyl group by a hydrogen (43). The resultant molecule is more potent than daunorubicin and doxorubicin and active by both the iv and oral routes (3,44). In the murine model systems idarubicin is superior to the earlier compounds in the treatment of leukemias, but, overall, is inferior to doxorubicin in solid tumors. It was advanced to clinical trials because of its favorable activity in leukemia, activity by the oral route, and a considerably improved cardiotoxic index compared to the earlier compounds. The clinical trials to date have demonstrated the drug to be active in acute leukemia and to show promise in breast cancer, particularly by the oral route; however, idarubicin will probably prove to be inactive in the majority of solid tumor varieties.

In phase I trials, myelosuppression was the dose-limiting toxic effect; leukopenia was more severe than thrombocytopenia. The suggested phase II doses were 12.5-15 mg/m$^2$ iv and 40-50 mg/m$^2$ orally at 21-day intervals (45). Nausea and vomiting were mild with iv idarubicin but of moderate intensity with the drug's oral administration. Alopecia was mild; stomatitis was uncommon. These phase II dosage projections and predictions regarding toxicity have proven reliable in the drug's assessment to date in solid tumors.

#### *Clinical Pharmacokinetics and Metabolism*

Idarubicin is cleared from the plasma in a multiexponential fashion, with an initial phase half-life ranging from 2 to 14 minutes and a terminal phase ranging from 3 to 45 hours. Idarubicinol is the only metabolite detected to date; it is formed extensively and retained in the plasma for a prolonged period, with a projected half-life > 48 hours (45-47). Whether the drug is given by the iv or oral route, the AUC of idarubicinol exceeds that of the parent compound. This is of considerable interest in

that idarubicinol has cytotoxic and antileukemic activity equal to that of idarubicin (48). Accordingly, the metabolite may make a major contribution to the biologic effects observed in patients. In multiple-day dosing, as used in patients treated for acute leukemia, the prolonged plasma half-life of idarubicinol leads to that metabolite's accumulation in plasma over the several days of therapy (49). The bioavailability of idarubicin by the oral route is approximately 30% that of the iv drug; this pharmacokinetic observation is in accord with the observed differences in the myelosuppression by the two routes (47).

#### *Studies in Acute Leukemia*

When given on iv schedules ranging from 8 to 15 mg/m$^2$/day × 3, idarubicin has demonstrable activity in previously treated patients with acute leukemia (49,50); the observed CR induction rates of 17% in acute nonlymphoblastic leukemia (ANLL) and 30% in acute lymphoblastic leukemia are comparable to other active single agents used in previously treated patients with leukemia. In a follow-up study, combining idarubicin with cytarabine (Ara-C), Berman et al (51) observed CR induction in eight of 34 heavily pretreated patients with ANLL. The drug has subsequently been advanced to a prospectively randomized comparison with daunorubicin as initial therapy for ANLL, each drug being given in combination with an identical regimen of Ara-C. Responses are being obtained with both treatment arms, but the patient numbers are too small at present to make analysis meaningful.

#### *Studies in Solid Tumors*

In contrast to a generally negative experience with solid tumors (*see* below), idarubicin has produced objective regression of advanced breast cancer in several European centers. Bonfante et al (36) reported CR plus PR in three of 22 patients following iv idarubicin and in five of 19 patients following oral administration, while Bastholt et al (52) observed responses in 13 of 26 patients treated by the oral route. In the latter study, the women were postmenopausal, with a median age of 69 years, and only two had received prior cytotoxic chemotherapy. Although this series of elderly, largely unpretreated women may be considered an unusually favorable group, the observation deserves expanded study.

In the preclinical murine transplanted tumor screen, idarubicin had been inferior to doxorubicin in the majority of the solid tumors; except for the above-described studies in breast cancer, the clinical experience to date has failed to document useful activity in human solid tumors. It should be noted, however, that the majority of the completed negative studies are in tumors which also respond poorly to doxorubicin. Negative phase II trials of idarubicin have been published in malignant lymphoma (in a doxorubicin-refractory population); non-small cell lung cancer by the iv and oral routes; colorectal, gastric, ovarian, and renal cancers; and melanoma.

#### *Cardiotoxic Effects*

The clinical evaluation of idarubicin has not yet defined a clear cardiotoxic dose. In the leukemia trials, which provide the most intensive drug usage and a population that frequently has received extensive prior anthracycline exposure, there has been only one case of heart failure on a presumed anthracycline-induced basis; it occurred in a patient who had received 426 mg/m$^2$ of daunorubicin before entering into the idarubicin trial.

### Carubicin

Carubicin was developed in the Soviet Union where the early clinical reports were encouraging (53); follow-up randomized comparison against epirubicin in breast cancer patients (15) and against doxorubicin in patients with soft tissue sarcomas (54) has failed to confirm activity of the drug in solid tumors. The drug's pharmacokinetic behavior in man is of interest in that it closely resembles that of daunorubicin and idarubicin, characterized by extensive formation of the 13-OH metabolite, which has a prolonged plasma half-life (55,56). Like idarubicin, from which it differs only by a 4-OH, carubicin is absorbed orally.

## ACLARUBICIN

Aclarubicin was isolated from culture broths of *Streptomyces galileus* by Oki et al (57). It represented a considerable departure from the daunorubicin and doxorubicin molecules both in regard to its modified chromophore (aklavinone), which lacks the C-11-OH, contains a methyl carboxylate ester at C-10, and an ethyl at C-9, and in its C-7 trisaccharide side chain containing, sequentially, rhodosamine, 2-deoxyfucose, and cinerulose. It was advanced to clinical trials because of its favorable spectrum of activity in transplantable murine leukemias and solid tumors (58,59), its low mutagenicity, and its apparently reduced cardiotoxicity (60). It is structurally closely related to marcellomycin, which has had only brief clinical evaluation.

### Cellular Pharmacology

In the initial metabolic evaluation of the drug's effects on cells in culture, aclarubicin demonstrated a more profound inhibitory effect on cellular RNA synthesis than on DNA synthesis; this presented a contrast from the pattern observed with daunorubicin and doxorubicin (59,61). More lipophilic than either doxorubicin or daunorubicin, and possessing a lower pKa than those analogs (resulting in a greater proportion of uncharged molecules at physiologic pH), aclarubicin enters cells

more rapidly than they do, with a pattern consistent with simple diffusion of the (uncharged) molecules through the lipid domain of the plasma membrane (62,63). Like daunorubicin and doxorubicin, its efflux from the cell is apparently energy-dependent. Within the cell its initial localization is 75%–90% within the nucleus (62,63). The extent of cellular catabolism of aclarubicin may vary with differing cell lines; however, extensive cytoplasmic deglycosidation to the aklavinone can be demonstrated in Friend erythroleukemia cells. This catabolism is accompanied by efflux of aclarubicin from the nucleus and a progressive loss of inhibitory effect.

In view of this drug's established therapeutic effectiveness against leukemia (*see* below), it is of interest that subcytocidal concentrations of aclarubicin and marcellomycin have been shown to produce differentiation of the human promyelocytic leukemia cell line HL-60 (64). The effect has been observed in cell culture and (to a considerably lesser degree) in diffusion chambers implanted into the peritoneal cavities of mice treated with these anthracyclines. Doxorubicin and daunorubicin produce cytotoxic effects in these assay systems but fail to produce differentiation.

### Clinical Pharmacokinetics and Metabolism

The clinical pharmacokinetics and metabolism of aclarubicin have been described by Egorin et al (65,66) and by Karanes et al (67). The parent compound is rapidly cleared from plasma; high-performance liquid chromatography analysis permitted measurements to 24 hours and generation of data which best fit a two-compartment model, with initial and terminal half-lives of 6.6 minutes and 13.3 hours, respectively (67). Two metabolites appeared and became the predominant circulating aclarubicin-derived species after the first hour following drug administration; they cleared with an apparent terminal-phase half-life of 25 hours (67). Egorin et al (65,66) identified the two circulating metabolites as "F1" or bisanhydroaklavinone and its demethylated derivative, bisanhydroaklavinic acid; these are probably noncytotoxic. The pattern that emerges is one of relatively rapid clearance of the parent compound from plasma and extensive metabolism of the drug to relatively inert molecules, not only within the liver but within somatic tissues, including some neoplastic cell varieties.

### Clinical Studies—Toxicity

Aclarubicin has been evaluated by a variety of parenteral schedules, beginning with a low dosage daily, progressing to a higher dosage at 21-day intervals. In patients with leukemia, the drug has been given at a chronic low daily dose of 14 mg/m$^2$/day × 7–20, or at higher doses in more acute courses, eg, 100–120 mg/m$^2$/day × 3. The usual dose-limiting toxic effect for most schedules has been myelosuppression, in which both granulocytes and thrombocytes were depressed. Gastrointestinal toxicity in the form of nausea and vomiting was also common; mucositis and diarrhea were seen somewhat less frequently. Significant alopecia was unusual by any schedule.

The cardiotoxic effects of aclarubicin have been examined both in the phase I studies and in the intense usage that accompanies therapy for acute leukemia. The drug does produce cardiotoxic effects, although the pattern differs significantly from that conventionally expected with doxorubicin. The frequency with which abnormalities were observed related to the intensity both of therapy and of observation. In patients studied with Holter monitors, Van Echo et al (68) recorded a significant increase in the frequency of atrial and ventricular premature beats following chemotherapy; they also noted instances of atrioventricular block. These changes are not unique to aclarubicin; similar occurrence has been reported in doxorubicin-treated patients followed by the Holter monitor technique. In leukemic patients having daily ECG tracings during the period of therapy, Warrell et al (69) noted the occurrence of ST- and T-wave abnormalities in 43% of the patients. Although isolated instances of congestive heart failure in aclarubicin-treated patients have been reported, as well as a persistent decrease in some patients' left ventricular ejection fraction on RNCA scan, the frequency appears low in relation to the population at risk. In the final analysis the relative degrees of cardiotoxicity of the several anthracycline analogs must be examined in the same fashion that is required to assess their comparative therapeutic efficacies, by means of prospectively randomized comparison in homogeneous treatment populations. The drugs should be administered either as single agents or as part of otherwise identical combination regimens in patients who are anthracycline-naive.

### Therapeutic Effects—Acute Leukemia

The observations of multiple centers in Japan, Europe, and the United States demonstrate that aclarubicin has clear utility in the treatment of ANLL (69–74). The observed CR rates of the drug as a single agent in previously treated patients have ranged from 13% to 44% and the observed durations of CR from 1 to 18 months.

In the initial reports there was no obvious relationship between the dosage schedule used and the CR rate obtained, provided that sufficient quantities of the drug were given to produce bone marrow hypoplasia. Recently, Machover et al (72) have reported an increased response rate with administration of aclarubicin in a series of 10-day courses of 15 mg/m$^2$/day, with 10-day intervals between courses. They observed a CR rate of 44% (11 of 25 patients) contrasted with a rate of 15% (two of 13) in patients treated with continuous therapy of 10–30 mg/m$^2$/day to a maximal dose of 300 mg/m$^2$. Although this observed difference, obtained in a sequential (non-

randomized) patient series, is of great interest, confirmation in a larger patient population with a prospectively randomized design is necessary before this schedule can be considered to be superior to other, more intensive, schedules.

Yamada et al (74) have recently summarized the results of a cooperative group study of aclarubicin in Japan. Using a dosage of 14 mg/m$^2$/day until bone marrow hypoplasia was obtained, they observed eight CRs in 21 previously untreated patients with acute leukemia (38%); this contrasted with seven CRs in 41 previously treated patients (17%). In a phase III extension of their studies, they combined aclarubicin (14 mg/m$^2$/day × 15) with behenolyl-Ara-C, 6-mercaptopurine, and prednisolone. This multidrug regimen produced CR rates of 67% in previously untreated patients and 63% in previously treated patients. The response rates in previously untreated patients are quite comparable to those achieved with current front-line regimens containing daunorubicin and Ara-C.

### Therapeutic Effects—Solid Tumors

In contrast to the established activity for aclarubicin in acute leukemia, the drug does not appear to have major activity in solid tumors. The initial studies in Japan, summarized by Ogawa (75), yielded no CRs and gave the following overall PR rates: 10.8%, breast cancer; 8%, gastric cancer; and 5.6%, non-small cell lung cancer. PR was not obtained in small numbers of patients with ovarian, colorectal, and small cell lung carcinomas.

Subsequently, negative reports have been published on clinical trials of aclarubicin in colorectal, head and neck, and renal cancers. More extensive, as yet unpublished studies carried out under the sponsorship of NCI have shown aclarubicin to have no meaningful activity in the following solid tumor varieties: lung (small cell and non-small cell), breast, head and neck, ovarian, and pancreatic cancers and plasma cell myeloma. The drug's activity in malignant lymphoma is not fully defined; reported response rates have ranged from 8% to 60%. Since these results may have been greatly influenced by prior exposure to doxorubicin-containing regimens, further study should focus on anthracycline-naive patients.

## MENOGARIL

Menogaril is a new anthracycline that has newly entered clinical trials. The compound is a semisynthetic derivative of nogalamycin (76,77) that was chosen for clinical trials because of an antitumor spectrum comparable to that of doxorubicin, lower cardiotoxicity in rabbits, activity by the oral and iv routes, and partial differences in biochemical effects from those of doxorubicin, suggesting a novel mechanism of action. The preclinical data have been reviewed by McGovern et al (78). Structurally, it has major differences from doxorubicin; these include a dimethylamine sugar attached at C-1 and C-2 through carbon-oxygen and carbon-carbon bonds, a methyl group at C-9 rather than a hydroxyacetal, a hydroxyl at C-4, and a hydrogen at C-11. Although menogaril exposure does produce DNA damage, it binds only weakly to DNA and has relatively little inhibitory effect on synthesis of RNA. More lipophilic than doxorubicin, it enters cells more rapidly and accumulates to higher concentrations than doxorubicin. In the initial clinical trials, myelosuppression has been dose-limiting; phase II assessment has recently begun.

## MITOXANTRONE

Mitoxantrone is a synthetic anticancer drug of the anthracenedione group. It resulted from studies of molecules with features promoting intercalation with DNA. This group of compounds has a planar quinoid chromophore with an intercalative capacity; the basic aminoalkyl side chain(s) are believed to stabilize the interaction with DNA. The development of mitoxantrone has been recently reviewed by White and Durr (79). It was discovered independently by two research groups (80,81) and found to have significant activity against a variety of transplanted tumors. The mechanism of action is not fully defined; although the drug binds tightly to purified DNA in an intercalative manner and has a nuclear location in intact cells, it may bind to nuclear DNA on an electrostatic basis. It inhibits both DNA and RNA synthesis and produces DNA strand breakage in treated cells. In comparison to doxorubicin, the drug has only weak stimulatory effects in free-radical-generating systems (82). The plasma clearance of the drug is triexponential, with a prolonged terminal half-life and extensive retention of the drug within tissues (83). Four metabolites have been detected, one of which is the dicarboxylic acid derivative of mitoxantrone (84). Extensive clinical studies have demonstrated the drug to have useful activity against breast cancer and acute leukemia. Mitoxantrone produces significantly less nausea and vomiting and alopecia than doxorubicin; although it may not be devoid of cardiotoxic potential, it is less than that of doxorubicin in randomized comparative studies.

### Clinical Studies

The most widely studied dosage schedule has been an intermittent dose at 21-day intervals; 12–14 mg/m$^2$ is commonly compared to doxorubicin at 60–75 mg/m$^2$. The acute dose-limiting toxic effect is leukopenia. In prospectively randomized trials in breast cancer patients, mitoxantrone is significantly less emetogenic than doxorubicin and markedly less likely to produce alopecia (85). In phase II studies mitoxantrone has produced isolated instances of myocardial damage; however, these events have usually occurred in the context of prior anthracycline exposure (86). In the collected experience of the

TABLE 3.—Prospectively randomized comparison of mitoxantrone with doxorubicin in patients with advanced breast cancer

| Study population | Investigators or group | Regimen* | No. of evaluable patients | No. of patients with CR + PR (%) |
|---|---|---|---|---|
| Breast cancer, previously treated, anthracycline-naive | SWOG | Mitoxantrone (14 mg/m$^2$) | 36 | 6 (17) |
| | | vs | | |
| | | Doxorubicin (60 mg/m$^2$) | 35 | 9 (26) |
| Breast cancer, previously treated, anthracycline-naive | Allegra et al | Mitoxantrone (14 mg/m$^2$) | 89 | 22 (24) |
| | | vs | | |
| | | Doxorubicin (75 mg/m$^2$) | 83 | 29 (35) |
| Breast cancer, previously untreated and previously treated, anthracycline-naive | Bennett et al | CNF† | 38 | 16 (42) |
| | | vs | | |
| | | CAF‡ | 53 | 24 (45) |

* Every 21 days.
†CNF = cyclophosphamide (500 mg/m$^2$), novantrone (10 mg/m$^2$), and 5-FU (500 mg/m$^2$).
‡CAF = cyclophosphamide (500 mg/m$^2$), doxorubicin (50 mg/m$^2$), and 5-FU (500 mg/m$^2$).

multiple comparative studies in anthracycline-naive breast cancer patients, a dose-related occurrence of heart failure was not noted in the mitoxantrone patients but was evident in the doxorubicin arm (85).

### Therapeutic Effects in Breast Cancer

Therapeutic effects of mitoxantrone were detected in the phase II evaluation of the drug, with collective rates of 33% in patients with no prior chemotherapy and 14% in previously treated patients (87). The drug has subsequently been examined in a broad series of collaborative trials, the reports of which were collected in a recent issue of Investigational New Drugs. These studies confirmed the original observations in single-arm assessment; 49 of 155 patients (32%) receiving mitoxantrone as initial chemotherapy for advanced breast cancer achieved CR or PR (88,89). Mitoxantrone was also compared with doxorubicin and bisantrene in single-agent usage (90,91), as well as a component of a multidrug regimen, again versus doxorubicin, both drugs being given in combination with cyclophosphamide and 5-FU (92). The preliminary results of these studies are presented in table 3.

In the single-agent comparisons, mitoxantrone gave CR plus PR rates of 17% and 24%, compared to 26% and 35% for doxorubicin. Although the severity of myelosuppression was comparable, mitoxantrone produced less vomiting and alopecia. The initial response rates for CNF versus CAF were quite comparable (42% vs 45%), but the two series are still quite small.

### Effects in Acute Leukemia

Induction of CR in patients with acute leukemia was demonstrated in the early trials with mitoxantrone and confirmed in a multicenter study (93). When given on a 5-day schedule at either 10 or 12 mg/m$^2$/day, 11 of 32 patients with ANLL achieved CR; remission was obtained in only one of 14 patients with ALL. Cardiac changes were seen in ten of 70 patients entered in the study; a causal relation to the mitoxantrone therapy is possible but the issue is confounded by prior anthracycline therapy and concomitant severe stress associated with the underlying illness.

### Effects in Other Tumors

Mitoxantrone has produced CR and PR in heavily pretreated patients with malignant lymphoma, with an overall response rate of 43% in 69 patients with non-Hodgkin's lymphoma who received the drug at a dose of 14 mg/m$^2$ at 21-day intervals. This encouraging result merits further exploration. Based on the results of extensive series, mitoxantrone does not have meaningful antitumor activity against the following cancer varieties: sarcomas; carcinoma of the head and neck, stomach, large bowel, lung (non-small cell), kidney, or prostate; or melanoma.

## DISCUSSION

There are several common threads which run through the preclinical characteristics of the anthracycline analogs that have been taken into clinical trial. In general, they have been selected because they have demonstrated a decrease in toxic effects in model systems without an appreciable loss of antitumor effectiveness. In particular, a reduction in cardiac toxicity has been considered a prime factor in promotion of a candidate to clinical trial. The antitumor effectiveness in the model systems has been retained, but it was not enhanced. Although a candidate agent may have been superior to doxorubicin in an individual tumor system, none has shown broad superiority across the solid tumor spectrum. In greater or lesser degree, the drugs that have been pro-

TABLE 4.—Comparative clinical antitumor spectrum of anthracyclines

| Anthracycline | Relative lipophilicity | Degree of alopecia | Therapeutic effectiveness for diagnostic group | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Acute leukemia | Non-Hodgkin's lymphoma | Breast | Ovary | Sarcoma | Gastric |
| Doxorubicin | Doxorubicin | Doxorubicin | +++ | +++ | +++ | +++ | ++ | + |
| Epirubicin | = Doxorubicin | < Doxorubicin | ? | ++ | +++ | ++ | + | + |
| Esorubicin | > Doxorubicin | < Doxorubicin | ? | ? | ± | ? | — | ? |
| THP-ADR | > Doxorubicin | < Doxorubicin | ? | + | ? | + | ? | ± |
| Daunorubicin | > Doxorubicin | = Doxorubicin | +++ | ± | — | — | — | — |
| Idarubicin | >> Doxorubicin | < Doxorubicin | +++ | ± | +* | — | — | — |
| Aclarubicin | > Doxorubicin | << Doxorubicin | +++ | ± | — | — | — | — |
| Mitoxantrone | > Doxorubicin | << Doxorubicin | +++ | ++ | +++ | ? | — | — |

*Orally.

moted to clinical trial have been more lipophilic than doxorubicin, crossing the cell membrane more rapidly both on influx and efflux. The comparative clinical antitumor spectrum of nine of the analogs is presented in table 4.

All of the analogs examined to date, where there has been adequate assessment, have proven to be active in the treatment of acute leukemia. Three of these, idarubicin, aclarubicin, and mitoxantrone, would be appropriate candidates for prospective randomized comparison with daunorubicin in otherwise identical combination regimens as initial therapy for acute leukemia. In view of the above, the preclinical screens appear reasonably accurate in selecting agents that are active in patients with acute leukemia. There is considerable structural diversity among the active compounds; accordingly, the antileukemic activity is not readily expressed in terms of structure-activity relationships.

At the other extreme, none of the new compounds has shown therapeutic effects in doxorubicin-nonresponsive tumor varieties such as non-small cell lung cancer, kidney cancer, melanoma, etc. This should not be surprising; it is in accord with the behavior of the analogs in the transplanted tumor systems. It is in the doxorubicin-responsive tumors where some insight into structure-activity relationships may be found. Among these, breast and ovary cancer and non-Hodgkin's lymphoma are chemoresponsive tumors; sarcoma and gastric cancer are relatively resistant.

In the studies to date only epirubicin, which presents a minimal modification from doxorubicin, has demonstrable activity in sarcomas and gastric cancer; in the sarcomas it may not be quite as active as doxorubicin when the two are given at equal dosage. Esorubicin, idarubicin, aclarubicin, and mitoxantrone have been negative in these tumor systems. Once again, there is sufficient structural diversity among the negative compounds that it is difficult to construct a hypothesis merely on clinical grounds. It would be useful in this regard to obtain more detailed data on the performance of these clinically studied analogs in the model systems of great current research interest in regard to anthracycline effects, ie, inhibition of topoisomerase II and the generation of hydroxyl radicals and membrane damage in the presence of iron and free sulfhydryls.

Regardless of the present inability to predict increased anticancer effectiveness, the available data suggest that the animal models for chronic cardiotoxicity do provide rather sound guidance for the drug's clinical behavior. It is evident that none of the anthracycline analogs is likely to be totally devoid of cardiotoxic effect; nevertheless, the detailed data being derived from the prospective randomized comparison studies in breast cancer strongly suggest that both epirubicin and mitoxantrone have significantly reduced cardiotoxicity of clinical significance, and that fact may provide an increase in the therapeutic index of those drugs.

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# Relation of Preclinical Toxicology to Findings in Early Clinical Trials

**Charles K. Grieshaber[1,*] and Silvia Marsoni[2]**

**The evolution of the Division of Cancer Treatment's preclinical toxicology protocols over the last decade, a description of the current protocol, and the progress of this protocol regarding quantitative and qualitative relationships to current clinical findings are presented in this report. Data are reviewed for seven experimental antineoplastic drugs. Preliminarily, the following conclusions can be made from preclinical and clinical toxicology experience with these first seven drugs. The $\frac{1}{10}$ MELD10 establishes a safe human starting dose. Six of seven drugs could have started at $\frac{1}{10}$ MELD10 with no adverse effects. Toxicity data from the beagle dog did not effectively predict whether the $\frac{1}{10}$ MELD10 dose level represents a hazard to humans. Clinical dose escalation procedures can not be efficiently predicted from currently acquired preclinical information. Toxicity studies in dogs were effective in disclosing the human dose-limiting toxic effects and approximated the maximally tolerated dose for six of seven drugs. In the exceptional case, the dose level predicted for human toxicity was grossly underestimated in both experimental species. We conclude that the preclinical toxicity data from the beagle dog are valuable in predicting the potential risk to humans, even though the safety of the entry-level doses is occasionally underpredicted.**
**[Cancer Treat Rep 70:65–72, 1986]**

Cancer, with few exceptions, is a lethal and refractory disease. New agents are constantly being sought and brought to clinical testing in efforts to discover treatments for this devastating disease. These experimental anticancer drugs are powerful pharmacological tools which exhibit an extremely narrow therapeutic index. Typically with antineoplastic agents, biologically inactive doses may be within 1 log of lethal doses (1-3). Thus, early clinical trials (phase I) of anticancer agents are conducted in patients with a short life expectancy due to advanced, intractable disease, rather than in healthy human volunteers. The establishment of a reliable method for determining a safe, though not homeopathic, starting dose in humans has been a primary goal of preclinical toxicologists and clinical pharmacologists involved in anticancer drug development. To this end, preclinical animal toxicology studies are conducted by the National Cancer Institute (NCI) to provide quantitative and qualitative information that establishes a safe clinical entry dose and predicts potential acute and subacute hazards of compounds introduced in phase I clinical trials. Approximately six to eight compounds are tested in the NCI preclinical toxicology program annually.

With the inception of the Drug Development Program at the NCI in the mid-1950s, preclinical animal toxicology studies have been performed on all candidate agents. These toxicology studies routinely employed single- and multiple-dose treatment schedules in mice, rats, dogs, and monkeys.

In 1973, the Laboratory of Toxicology, Division of Cancer Treatment, NCI, published its guidelines for toxicological evaluation of anticancer drugs (4). The complex protocol is outlined in figure 1. Essentially, a lethal dose was established in dogs and monkeys from which nonlethal but toxic doses were derived. These studies established the acute toxic effects of the drugs in multiple biological systems. Longer-term effects and effects of repeated administration elicited by the drugs were assessed solely in dogs. At the end of the required observation periods, surviving animals were sacrificed for full histopathologic evaluation. Starting doses for clinical trials were generally derived by calculating one-third of the lowest toxic dose for the most sensitive species (dog or monkey) if the long-term and schedule-dependency studies assured safety at these dose levels.

In the late 1970s, the accumulation of a large human and animal toxicology data base permitted extensive interspecies comparison of the adverse effects of antineo-

[1]Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.
[2]Laboratory of Clinical Pharmacology, Mario Negri Institute, Milan, Italy.

*_Reprint requests to:_ Dr. Charles K. Grieshaber, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Landow Bldg, Rm 5B06, National Institutes of Health, Bethesda, MD 20892.

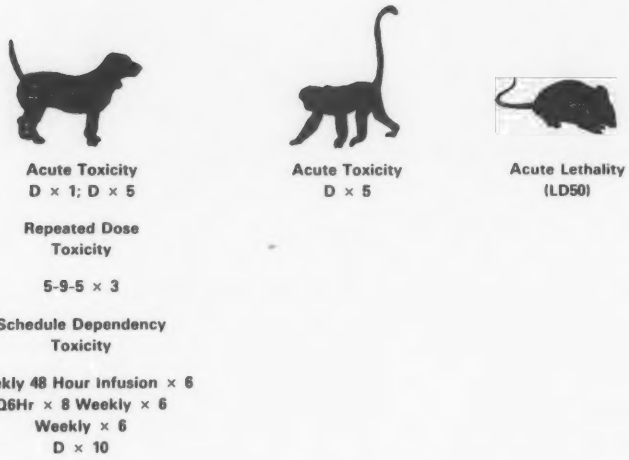


FIGURE 1.—NCI preclinical toxicology protocols (1973-1980).

plastic agents. Following careful evaluation, proposals designed to simplify and streamline the preclinical toxicity testing of antineoplastics were developed. In October 1979, the Food and Drug Administration Oncologic Drugs Advisory Committee concurred with the concept that preclinical studies in the mouse could predict quantitative drug toxicity in man. Guarino et al (5) stimulated this view. Further bases for this concept were published a few years later (6). The starting dose for clinical trials was established as $\leq \frac{1}{10}$ the LD10 computed from statistically reliable murine lethality studies. The Committee also recommended that an additional experimental species be tested to assess the safety of proposed human starting doses and to predict qualitative organ toxicity. The beagle was deemed the best choice due to the extensive background information available.

In 1980, toxicology protocols were developed by the Division of Cancer Treatment incorporating these recommendations. These protocols (figure 2) included lethality studies (daily × 1, daily × 5) in BALB/c/DBA2 $F_1$ hybrid mice and toxicity studies (daily × 1, daily × 5) in beagle dogs. Toxicity studies (daily × 1, daily × 5) in mice were added as options to determine whether a useful model for human toxicity could be developed using this species.

FIGURE 2.—NCI preclinical toxicology protocols (1980).

Since 1981, 16 compounds have undergone animal toxicology testing in dogs and mice under the new preclinical toxicology protocols. Seven of these compounds have completed phase I evaluation in man at the time of this evaluation. In this paper, we present comparisons of drug-induced animal toxicity to that assessed in humans to evaluate the accuracy and reliability of current animal models for providing an initial clinical dose for phase I trials, establishing its safety, and predicting the primary organ systems most likely at risk in humans.

## MATERIALS AND METHODS

### Preclinical Toxicology Protocol

The specific details of the preclinical animal toxicology protocols are published elsewhere (7). Procedures leading to the estimation of the human starting dose are summarized here with a brief description of acute qualitative organ toxicity findings in dogs.

The proposed human starting dose is derived from lethality studies in mice, then tested for safety in dogs (fig 3). The LD10, LD50, and LD90 are established, then converted to a body-surface area equivalent. The pivotal dose is the $\frac{1}{10}$ LD10 equivalent, $\frac{1}{10}$ MELD10. This dose level is tested in dogs to ascertain whether it produces toxicity. If severe toxicity is observed in dogs, further testing is performed, de-escalating the dose until minimal or no toxicity is found. In such cases, the initial human doses would be based on the human equivalent ($mg/m^2$) of one-third of the dog dose experimentally determined to cause minimal reversible toxicity or on a fraction of the $\frac{1}{10}$ MELD10.

To establish the upper bracket of the toxicity curve, the MELD10 is also tested in the dog. If this dose does not produce overt, reversible toxicity in the dog, the dose is

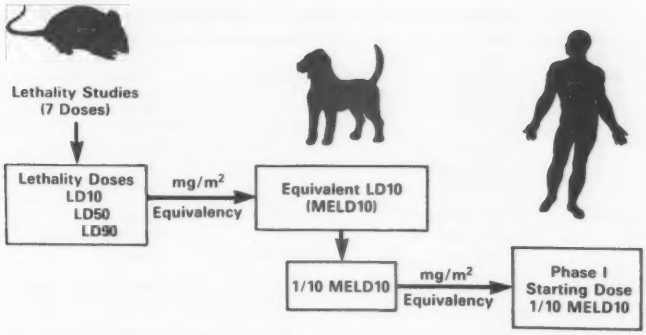


FIGURE 3.—Current NCI toxicology protocol summary of dose estimation scheme.

systematically increased until overt toxicity is encountered. Clinical chemistry and macroscopic and microscopic pathology assessment are performed in both species. In both experimental species, compounds are tested with single and daily × 5 schedules, the drug given via bolus injection unless otherwise indicated.

### Phase I Protocols

All human data have been generated in phase I trials conducted under the sponsorship of the NCI. The primary source of information is the CTMS Data Base of the NCI, a computerized collection of demographic, physical examination, clinical chemistry, autoptic data, and physician observations pertaining to all patients participating in phase I trials of the NCI.

## RESULTS

The 16 agents shown in table 1 have had preclinical toxicology studies conducted under the current protocol. An Investigational New Drug Application has been submitted for each drug. At the time of this evaluation, seven drugs have completed phase I clinical trials, while nine others are in phase I evaluation.

TABLE 1.—Antineoplastic drugs evaluated under the new preclinical toxicology protocol

| NSC No. | Drug |
|---|---|
| | ***Completed phase I evaluation*** |
| 264880 | DHAC |
| 296934 | Teroxirone |
| 241240 | Carboplatin |
| 280594 | Triciribine |
| 141633 | Homoharringtonine |
| 3051 | *N*-Methylformamide |
| 312887 | Fludarabine |
| | ***Ongoing phase I evaluation*** |
| 286193 | Tiazofurin |
| 172112 | Spiromustine |
| 305884 | Acodazole |
| 253272 | Caracemide |
| 269148 | Menogaril |
| 526417 | Echinomycin |
| 322921 | Pibenzimol |
| 125973 | Taxol |
| 352122 | Trimetrexate |

### Quantitative Toxicology

The entry-level doses for phase I trials for each of the drugs as a fraction of the MELD10 are shown in table 2. Although seven drugs are under review, some were started at different fractions of the MELD10 depending upon the schedule of administration; thus, five drugs on seven schedules were started at $\frac{1}{10}$ MELD10. The entry-level dose was reduced to $< \frac{1}{10}$ MELD10 for four drugs: homoharringtonine (daily × 5), fludarabine (daily × 5), triciribine (daily × 1, daily × 5), and dihydro-5-azacytidine (DHAC) (daily × 5). The starting dose was found safe in humans with three of these drugs; the exception was fludarabine. Myelosuppression was noted in patients on both schedules following administration of the prescribed entry dose of fludarabine.

The toxic effects found in dogs at $\frac{1}{10}$ MELD10 during preclinical studies with homoharringtonine, triciribine, and DHAC are shown in table 3. It is clearly evident that serious toxicity was found in dogs for each drug at $\frac{1}{10}$ MELD10, ranging from effects on the alimentary tract with homoharringtonine and triciribine to myelosuppression with all three drugs. Histopathologic lesions in kidney, bladder, and prostate were also found in dogs after treatment with DHAC at $\frac{1}{10}$ MELD10. Furthermore, dosing of dogs with triciribine at ½ MELD10 on both schedules resulted in liver toxicity which was not completely reversible. Significant toxicity was evident in these animals to warrant moribund sacrifices of one dog on each schedule. As a result of these preclinical findings, starting doses for these three drugs in humans were lowered to $\frac{1}{30}$ or $\frac{1}{60}$ MELD10 as illustrated.

It is noteworthy that in clinical trials with these three drugs, no toxic effects were observed at entry doses nor was toxicity found upon dose escalation from the starting level to the dose equivalent to $\frac{1}{10}$ MELD10, except for mild to moderate nausea with DHAC (table 4). If the

TABLE 2.—Human toxicity observed at clinical entry doses

| Starting dose* | Drug | Schedule | Human toxicity |
|---|---|---|---|
| 1/10 | *N*-Methylformamide | Daily × 5 | None |
| | Carboplatin | Daily × 1, Daily × 5 | None |
| | Teroxirone | Daily × 1, Daily × 5 | None |
| | Homoharringtonine | Daily × 1 | None |
| | Fludarabine | Daily × 1 | Myelosuppression |
| 1/30 | Homoharringtonine | Daily × 5 | None |
| | Fludarabine | Daily × 5 | Myelosuppression |
| | Triciribine | Daily × 1, Daily × 5 | None |
| 1/60 | DHAC | Daily × 5 | None |

* Fraction of MELD10.

number of additional trial escalation steps required to achieve the maximum tolerated dose (MTD) in humans is analyzed, the additional steps range from two for triciribine to four for the other two drugs. Thus, findings of serious toxicity in dogs at the 1/10 MELD10 prospectively mandated several unnecessary escalation steps in clinical trials. Retrospectively, it would have been far more efficient to initiate human trials with these three drugs at the 1/10 MELD10 dose.

## Qualitative Toxicology

Since our qualitative animal toxicity studies are designed to prospectively define specific organ toxicity in man, evaluation of the "correctness of prediction" of animal data must come from retrospective comparison with toxic effects actually observed in man, particularly the dose-limiting toxic effects (DLTs).

Table 5 presents the major human DLTs for the seven drugs under consideration. The appearance or nonappearance of equivalent effects in dogs from either clinical parameters and/or histopathology analyses is also indicated. For the most part, myelosuppression or hepatotoxicity limited the clinical dosages, except for teroxirone, where phlebitis limited the dose of administered drug. All human DLTs capable of being assessed in dogs were seen at one or more doses tested in this species. The hypotension noted in humans following homoharringtonine administration was not predicted, as the preclinical protocol did not include blood pressure measurement. Likewise, subjective chest pain cannot be assessed in experimental animals.

Comparisons of MTDs at which human DLTs were noted with doses evoking corresponding toxic effects in dogs are shown in table 6. There is relatively good correlation between the human MTD and the lowest dose at which the similar toxicity was noted in dogs for carboplatin, homoharringtonine, and triciribine on the daily × 1 schedule. Poorer correlations are seen for teroxirone, in which the dog dose producing phlebitis and leukopenia is sixfold lower than the MTD, and fludarabine, where the dog dose eliciting myelosuppression was 20-fold higher than the MTD. Good interspecies correlation is noted relating doses to toxic effect for *N*-methylformamide, carboplatin, teroxirone, and homoharringtonine on the daily × 5 schedule. Poorer correlations are found for

TABLE 3.—Toxic effects observed in dogs at 1/10 MELD10

| Drug | Schedule | Toxic effect | Lowest dose observed | Human SD* |
|---|---|---|---|---|
| Homoharringtonine | Daily × 5 | Emesis, diarrhea, decreased wbc count | 1/10 MELD10 | 1/30 |
| Triciribine | Daily × 1 | Bloody stool, testicular atrophy | 1/10 MELD10 | 1/30 |
| | | Lethal (1), liver function tests nonreversible | ½ MELD10 | |
| | Daily × 5 | Diarrhea, bone marrow hypocellularity | 1/10 MELD10 | 1/60 |
| | | Lethal (1), liver function tests nonreversible | ½ MELD10 | |
| DHAC | Daily × 5 | Degeneration and epithelial erosion in kidney, bladder, prostate; bone marrow atrophy | 1/10 MELD10 | 1/60 |

* SD = starting dose as fraction of MELD10.

TABLE 4.—Human toxicity observed at 1/10 MELD10 for drugs started at < 1/10 MELD10

| Drug (schedule) | Starting dose* | Human toxicity at starting dose | Human toxicity at 1/10 MELD10 | Additional escalations |
|---|---|---|---|---|
| Homoharringtonine (daily × 5) | 1/200 | None | None | 4 |
| Triciribine (daily × 1; daily × 5) | 1/50 | None | None | 2 |
| DHAC (daily × 5) | 1/100 | None | Mild nausea | 4 |

* Fraction of MELD10.

DHAC, where the dose producing nausea and vomiting and myelosuppression in dogs is about 1/10 the MTD; for triciribine, where the dog dose producing liver toxicity was threefold higher; and for fludarabine, where the dog dose causing myelosuppression was 55-fold greater than the MTD.

Similar comparisons made between the major human DLTs and toxicity findings from studies in mice reveal that the DLT was predicted for three of five drugs on the daily × 1 schedule and for six of the seven drugs on the daily × 5 schedule (table 7). No evidence of myelosuppression was noted in mice upon treatment with carboplatin. Hepatotoxicity was not noted in mice at the dose levels of triciribine tested on either schedule.

Table 8 shows the corresponding human DLT in mice with the human MTD for each drug. The DLTs were not predicted for carboplatin or triciribine on the daily × 1 schedule. There was good dose correlation on this schedule for myelosuppression with homoharringtonine and poor dose correlation for leukopenia with teroxirone (mouse dose is 12-fold low) and fludarabine (mouse dose producing myelosuppression is approximately ninefold higher than the human MTD). The human DLT of liver toxicity for triciribine was not predicted in mice on the daily × 5 schedule. There is relatively good correlation between human and mouse doses for *N*-methylformamide hepatotoxicity and myelosuppression caused by carboplatin and homoharringtonine on this schedule. However, poor interspecies toxic dose correlations are found for DHAC (mouse dose causing myelosuppression is twofold low), for teroxirone (mouse dose causing leukopenia is fivefold low), and for fludarabine (mouse dose causing myelosuppression is 30-fold higher than the human MTD).

TABLE 5.—Corresponding toxicity between dog and man

| Drug (schedule) | Human DLT | Dog* | |
|---|---|---|---|
| | | Clinical parameters | Histopathology |
| *N*-Methylformamide (daily × 5) | Hepatotoxicity | + | + |
| Carboplatin (daily × 1; daily × 5) | Myelosuppression | + | + |
| Teroxirone (daily × 1; daily × 5) | Phlebitis | - | + |
| | Leukopenia | + | + |
| Homoharringtonine (daily × 1; daily × 5) | Hypotension | NA | NA |
| | Myelosuppression | + | + |
| Fludarabine (daily × 1; daily × 5) | Myelosuppression | + | + |
| Triciribine (daily × 1; daily × 5) | Hepatotoxicity | + | + |
| | Hyperglycemia (daily × 1) | + | NA |
| DHAC (daily × 5) | Chest pain | NA | NA |
| | Nausea and vomiting | + | NA |
| | Myelosuppression | + | + |

* NA = not assessed.

## DISCUSSION

A retrospective evaluation of findings in preclinical toxicology studies compared to dose-limiting adverse effects in humans is presented in this paper. To date, complete information is available for seven experimental antineoplastic agents. The following conclusions are made from quantitative toxicology experience with these first seven drugs. The murine derived 1/10 MELD10 provides a safe human starting dose. In fact, six of the seven drugs could have started at 1/10 MELD10 with no adverse effects in phase I patients. Toxicity data from studies in the beagle dog had little utility in predicting whether 1/10 MELD10 represented a hazard to humans. For example, data from dog studies indicated that the clinical starting dose should be lowered to a fraction < 1/10 of MELD10 for three drugs; however, there was little or no human toxicity noted upon escalation to 1/10 MELD10. With fludarabine, where the safe entry-level dose should have been < 1/10 MELD10, data from dog studies did not so indicate. Thus, with four of seven drugs, the safety of the clinical entry dose was incorrectly determined.

The quantitative toxicology analysis of fludarabine deserves more discussion. The first phase I trial explored the single-dose every-3-week schedule. Six patients were entered at 260 mg/m$^2$ (equivalent to 1/10 MELD10). Severe neutropenia was observed in five patients. Neutropenia and lymphopenia were observed in preclinical dog studies at doses of ⩾ 5248 mg/m$^2$ (2 × MELD10). Unpublished information (L. Malspeis, personal communication) suggests that the metabolism and pharmacokinetics of fludarabine in dogs and man may differ substantially. The total-body clearance of 2-fludarabine in the beagle is

TABLE 6.—Corresponding toxicity between dog and man

| | | Human | | | |
|---|---|---|---|---|---|
| | | MTD ($mg/m^2$/day) | | Dog dose* ($mg/m^2$/day) | |
| Drug | DLT | Daily × 1 | Daily × 5 | Daily × 1 | Daily × 5 |
| Carboplatin | Myelosuppression | 440-500 | 99 | 312 | 60 |
| Teroxirone | Phlebitis | 2250 | 400 | 360 | 320 |
| | Leukopenia | 2250 | 400 | 360 | 160 |
| Homoharringtonine | Hypotension | 9 | 5 | NA | NA |
| | Myelosuppression | 9 | 5 | 6.4 | 2.9 |
| Fludarabine | Myelosuppression | 260 | 40 | 5200 | 2200 |
| Triciribine | Hepatotoxicity | 250 | 55 | 338 | 154 |
| | Hyperglycemia | 250 | — | 676 | — |
| *N*-Methylformamide | Hepatotoxicity | — | 1170 | — | 1324 |
| DHAC | Chest pain | — | 2500 | — | NA |
| | Nausea and vomiting | — | 2500 | — | 223 |
| | Myelosuppression | — | 2500 | — | 223 |

*NA = not assessed.

three times that of humans. In addition, fludarabine undergoes metabolic deamination to the inactive, nontoxic 2-fluorohypoxanthine in dogs. Deamination occurs to a lesser extent in mice. However, the extent of deamination in the mouse is more comparable to that in humans and yet the human appears to be more sensitive to toxic effects of the drug. Collins et al (8) propose that this finding is the result of differences in target cell specificity. An accumulation of adenosine metabolites (similar to that with pentostatin) is a possible reason for human sensitivity to the drug.

The qualitative toxicologic findings in both dogs and mice compared to clinical effects can be summarized as follows. The human DLTs for the seven drugs on both administration schedules were observed in at least one dose level in dogs. For ten human DLTs which could be assessed in dog studies, all were predicted. In mice, of nine assessable human DLTs, six were predicted. A mathematical approach to this evaluation is presented in table 9. The ratio of human MTDs to the lowest dose at which comparable toxic effects were observed in animals is calculated to illustrate the dose level comparability or discrepancy. A ratio of $< 1.0$ indicates the toxic effect occurred in man at a lower dose than in experimental animals. A value of $> 1.0$ indicates the toxic effect occurred in experimental animals at a lower dose than in humans. Ratios between 0.3 and 3.0 are considered ideal, in that serious toxicity in humans would be gradually observed during the careful dose escalation in phase I trials, and that historically, one-third the toxic dose level (TDL) in dogs is usually safe for humans. In 12 drug schedules, the dog proved ideal (0.3-3.0) for dose-level prediction in nine. The mouse was nonpredictive for three schedules and the qualitative ratio was less than ideal for four others. These findings are graphically represented in figure 4. The preclinical toxicology studies in dogs revealed the human DLT 100% of the time with 75% accuracy in providing TDLs. The preclinical mouse studies revealed the DLT 75% of the time with 50% accuracy in predicting TDLs in the human.

Obviously, it is far better that experimental animals predict a human DLT at doses less than the MTD, since the clinician would be warned in advance of serious deleterious effects. Fludarabine is the only drug for which the dose level eliciting qualitative organ toxicity was severely underpredicted, that is, experimental animals showed myelosuppression at doses substantially greater than the human MTD. Whether this drug is pharmacologically unusual or a member of a group of drugs for

TABLE 7.—Corresponding toxicity between mice and man

| | | Mouse* | |
|---|---|---|---|
| Drug (schedule) | Human DLT | Clinical parameters | Histopathology |
| *N*-Methylformamide (daily × 5) | Hepatotoxicity | + | + |
| Carboplatin | | | |
| Daily × 1 | Myelosuppression | – | – |
| Daily × 5 | Myelosuppression | – | + |
| Teroxirone (daily × 1; daily × 5) | Phlebitis | – | – |
| | Leukopenia | + | + |
| Homoharringtonine (daily × 1; daily × 5) | Hypotension | NA | NA |
| | Myelosuppression | + | + |
| Fludarabine (daily × 1; daily × 5) | Myelosuppression | + | + |
| Triciribine (daily × 1; daily × 5) | Hepatotoxicity | – | – |
| | Hyperglycemia (daily × 1) | NA | NA |
| DHAC (daily × 5) | Chest pain | NA | NA |
| | Nausea and vomiting | NA | NA |
| | Myelosuppression | + | + |

*NA = not assessed.

TABLE 8.—Corresponding toxicity between mice and man

| Drug | DLT | Human MTD (mg/m²/day) | | Mouse dose* (mg/m²/day) | |
|---|---|---|---|---|---|
| | | Daily × 1 | Daily × 5 | Daily × 1 | Daily × 5 |
| Carboplatin | Myelosuppression | 440-500 | 99 | — | 120 |
| Teroxirone | Phlebitis | 2250 | 400 | — | — |
| | Leukopenia | 2250 | 400 | 180 | 80 |
| Homoharringtonine | Hypotension | 9 | 5 | NA | NA |
| | Myelosuppression | 9 | 5 | 13 | 5.8 |
| Fludarabine | Myelosuppression | 260 | 40 | 2340 | 1215 |
| Triciribine | Hepatotoxicity | 250 | 55 | — | — |
| | Hyperglycemia | 250 | 55 | NA | NA |
| *N*-Methylformamide | Hepatotoxicity | — | 1170 | — | 1262 |
| DHAC | Chest pain | — | 2500 | — | NA |
| | Nausea and vomiting | — | 2500 | — | NA |
| | Myelosuppression | — | 2500 | — | 960 |

*NA = not assessed.

which rodent and dog toxicity experiments are not predictive for humans must await future evaluation.

In light of the current preliminary evaluations, the dog is a better prognosticator for organ toxicity in humans than the mouse. It is noteworthy that preclinical protocols currently require toxicology studies in rats rather than in mice. In 1983, the optional mouse toxicity studies were replaced with toxicity studies in rats. This major modification was instituted due to a number of technical problems associated with studies in mice. Namely, sufficient blood could not be routinely collected for adequate clinical pathology measurements nor could serial sampling be done without compromising the experimental animals. By analysis of the comparative toxicity data on the seven drugs reviewed here, the mouse also does not provide as good a model for predicting toxic effects of antineoplastic drugs in humans as the dog.

As with all preliminary evaluations, however, additional information must be collected and thoroughly analyzed before a lasting, final conclusion can be reached. We must await completion of ongoing preclinical toxicology studies and clinical trials to ascertain whether the rat serves equally well or better than the dog as a predictive species for human toxicity.

TABLE 9.—Comparison of dose-limiting toxicity and MTD in man with doses in dogs and mice at which corresponding toxicity was observed

| Drug | Dose-limiting toxicity | Daily schedule | MTD man/LTD animal* | |
|---|---|---|---|---|
| | | | Man/dog | Man/mice |
| Carboplatin | Myelosuppression | Daily × 1 | 1.4 | Not predicted |
| | | Daily × 5 | 1.6 | 0.8 |
| Teroxirone | Myelosuppression | Daily × 1 | 6.2 | 12.5 |
| | | Daily × 5 | 2.5 | 5.0 |
| Homoharringtonine | Myelosuppression | Daily × 1 | 1.4 | 0.7 |
| | | Daily × 5 | 1.7 | 0.9 |
| Fludarabine | Myelosuppression | Daily × 1 | 0.05 | 0.11 |
| | | Daily × 5 | 0.02 | 0.03 |
| DHAC | Myelosuppression | Daily × 5 | 2.3 | 2.2 |
| Triciribine | Hepatotoxicity | Daily × 1 | 0.7 | Not predicted |
| | | Daily × 5 | 0.4 | Not predicted |
| *N*-Methylformamide | Hepatotoxicity | Daily × 5 | 0.9 | 0.9 |

*Ratio calculated from doses at which clinical observation (man and animal) and histopathological findings (dog, mouse) showed consistent toxic effects. < 1.0 = toxicity occurred in man at lower dose than in experimental animals; > 1.0 = toxicity occurred in experimental animals at lower dose than in man.

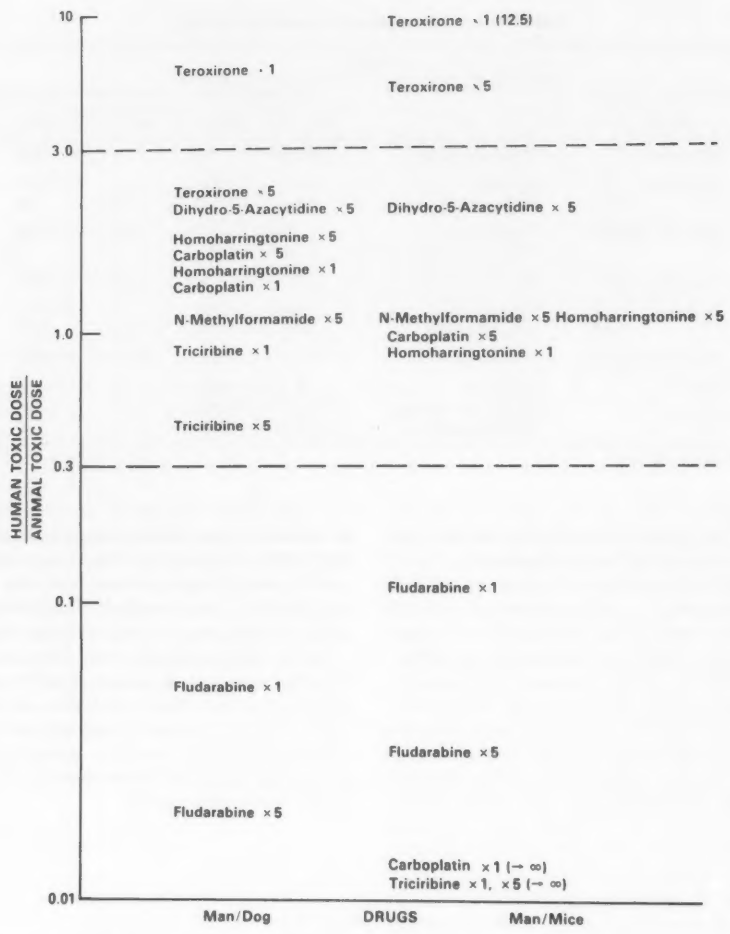


FIGURE 4.—Relationship of dose-limiting toxicity and MTD in man to doses in dogs and mice at which corresponding toxicity was observed. Horizontal lines bracket the "ideal" ratios. Man/mice ratio for carboplatin (daily × 1) and triciribine (daily × 1, daily × 5) equals infinity, since no corresponding human toxicity was observed in mice.

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# Potential Roles for Preclinical Pharmacology in Phase I Clinical Trials

Jerry M. Collins,[1,*] Daniel S. Zaharko,[1] Robert L. Dedrick,[2] and Bruce A. Chabner[1,3]

**Concepts elucidated from preclinical pharmacology studies have made a substantial impact on the clinical use of anticancer drugs. However, the majority of animal pharmacology results have not been available until after drugs have entered clinical trials. Since clinical pharmacokinetic measurements are already part of many phase I trials, human data could be directly compared with mouse data if mouse pharmacology studies were completed before clinical trials were initiated. Once the starting dose in a phase I clinical trial has been evaluated, subsequent doses are escalated until the maximum tolerated dose is reached. The rate of escalation is empirically defined by a modified Fibonacci series. This universal escalation scheme is applied to all drugs, with no modifications based upon pharmacology or other factors. If the starting dose is far removed from the maximum tolerated dose, a large number of dose escalations are required. Consequently, most patients receive subtherapeutic doses, and the amount of resources allocated to each drug increases. We are exploring potential strategies for controlling the rate of dose escalation based upon pharmacokinetic determinations in mouse and man. Retrospective analyses indicate that 20%–50% savings in the total number of dose escalations are possible. [Cancer Treat Rep 70:73–80, 1986]**

The preclinical pharmacology of anticancer drugs has been examined in a variety of species, especially the mouse, rat, dog, and monkey. Dr. Frank Schabel emphasized that there are many differences between man and animals that influence drug metabolism and, hence, antitumor activity and host toxicity. Some of the enzymatic and biochemical differences have been recognized, and there are probably many more unrecognized differences in drug activation, degradation, excretion, etc. He challenged preclinical cancer investigators to provide useful data to clinicians by being aware of the subtle differences that exist in determining anticancer drug activity in clinical and preclinical investigations (1).

Schabel and his colleagues at Southern Research Institute introduced a kinetic approach to the treatment of leukemias that permitted therapeutic extrapolations between mice and men. These investigators were also among the first to recognize the importance of concepts such as rates of disappearance of anticancer drugs from body fluids, minimum cytotoxic drug levels, and recovery rates of host tissues from courses of treatment. Use of such concepts in thorough studies with cytarabine in mouse leukemias, with subsequent extrapolation of such concepts to the clinic, made important contributions to the clinical use of drugs in the treatment of human leukemias (2,3).

As Skipper pointed out (4), the concept of the area under the concentration versus time curve (C×T) with regard to drug toxicity originated from the work of German pharmacologists during World War I (5). Their astute observation was that mustard agents are equally toxic whether a high concentration is inhaled for a short time or a low concentration is inhaled for a long time. The essential feature is that C×T is the determinant of effect rather than the absolute concentration itself. Scientists at Southern Research Institute devoted a large research effort to illustrate that this principle applies to anticancer agents within the limitations that result from cell heterogeneity and differences in mechanism of drug action (6,7). For example, antimetabolites whose action depends upon the inhibition of synthesis of macromolecular systems are more dependent on time of exposure above a certain threshold concentration than absolute concentration or C×T. This, no doubt, occurs because cells have discrete pe-

[1]Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

[2]Division of Research Services, National Institutes of Health, Bethesda, MD.

[3]The ideas and data presented in this paper have accumulated through the operations of the Blood Level Working Group of the Division of Cancer Treatment. The substantial contributions from the staff of the Developmental Therapeutics, Clinical Oncology, and Cancer Therapy Evaluation Programs have been essential to this work. Extramural contractors are now actively involved in the examination and application of these concepts.

*Reprint requests to:* Dr. Jerry M. Collins, Division of Cancer Treatment, National Cancer Institute, Bldg 10, Rm 6N119, National Institutes of Health, Bethesda, MD 20892.

riods of macromolecular synthesis prior to cell division which must be exposed to effective drug concentrations if cell division is to be affected. This team also stressed the importance of schedules of administration in planning and interpreting therapeutic trials of anticancer drugs. Along with Freireich et al (8), they observed that with many anticancer drugs, toxicity observations carry across species on a mg/m$^2$ basis, as long as schedules are similar. However, they also were aware that there are exceptions and that more complete exposure parameters such as C×T or plasma pharmacokinetics would allow more useful comparisons of toxic or therapeutic responses from experimental and clinical studies.

While these substantial contributions of preclinical pharmacology have led to improved clinical use of anticancer drugs, the results of most of these studies were not available until after the drugs had entered clinical practice. We would like to explore more immediate applications of preclinical pharmacologic information. In particular, to what extent does pharmacology determine the variations in toxicity which are observed between mice and men?

Although the mouse is an effective predictor of human toxicity on the average, its ability to predict for any particular drug is unreliable. If the pharmacologic causes of the variation in toxicity can be determined, we can try to use this knowledge to improve the way phase I trials are conducted. We can use it to make practical decisions which can reduce the number of individuals who are treated at nontherapeutic doses and also preserve patient resources so that a larger number of ideas can be clinically tested.

## Phase I Procedures

Although there is always therapeutic intent, the principal objective of phase I clinical trials is to determine the maximum tolerated dose (MTD), which will be used to establish the dose for more detailed efficacy studies or phase II testing. To begin human testing, a safe starting dose is needed. As reviewed earlier in this volume by Grieshaber and Marsoni (9), the current preclinical toxicology protocol does provide the basis for a safe starting dose, tailored to potency in rodents.

Finding a safe starting dose is only the first step in clinical testing. After the starting dose has been evaluated, subsequent doses are escalated until the MTD is reached. The process of dose escalation is governed by a *fundamental conflict*. On the one hand, there is a need to go slowly in order to avoid a sudden jump from no observable toxicity to a lethal dose; on the other hand, there is a need to go rapidly, so that large numbers of patients are not treated at ineffective doses.

Although the difficulties of an escalation which is too rapid are readily understood, the consequences of an escalation process which proceeds too slowly require some elaboration. One consequence is the effect on the *individual patient*. Each patient volunteers for a phase I study with the knowledge that the probability of success is not high. Our obligation is to maximize the chance that the dose which an individual receives has the potential for therapeutic value. If most of the effort in a phase I trial is spent evaluating doses that are eventually determined to be ineffective, we are not maximizing the chances of those patients treated at low doses.

Another area affected by slow escalation procedures is the overall progress of drug development. The entry of drugs to phase II testing (and beyond) is obviously slowed down, but there are other costs as well. In addition to a limited number of investigators, institutions, and other resources, there is a limited pool of patients who are eligible and willing to participate in phase I trials. In contrast to the limited set of patients and resources which is available, there is a large set of therapeutic strategies which could be evaluated. These strategies include both new drugs coming through the Decision Network and various approaches to the use of established drugs, such as new schedules or routes of administration, and the use of drug combinations or modulators. All ideas compete for the same pool of patients, and it is inefficient to tie up a large fraction of this pool in an escalation process that moves very slowly.

The compromise between the desire to escalate slowly enough to be safe and the desire to escalate fast enough to be efficient is known as the "modified Fibonacci" scheme (10). The initial escalation is rapid (100%, or doubling of the dose), and narrows down with successive increases until the 30%-35% range is reached (fig 1). For the moment, it is assumed that the human starting dose is $\frac{1}{10}$ of the mouse LD10, expressed on a mg/m$^2$ basis. Although the starting dose is tailored to the particular drug, the escalation process is not.

## Analysis of Phase I Results

How well does this strategy work? As Grieshaber and Marsoni (9) have shown, the number of escalation steps varies considerably. In figure 2, the drugs reviewed prospectively in the new preclinical toxicology protocol are listed in the second column. For three of the new drugs, there was additional species variation between the daily (× 1) dose and the doses given on 5 consecutive days (× 5). In the first column, ten other recent drugs are included as if they had been tested the same way. Rather than focusing on specific drugs at this stage, we simply want to emphasize the point that there is considerable variation, even though the *average* is quite reasonable.

Grieshaber and Marsoni (9) have noted that half of all drugs tested actually enter clinical trials at $\frac{1}{3}$ or less of the usual starting dose, that is, at $\frac{1}{30}$ or less of the

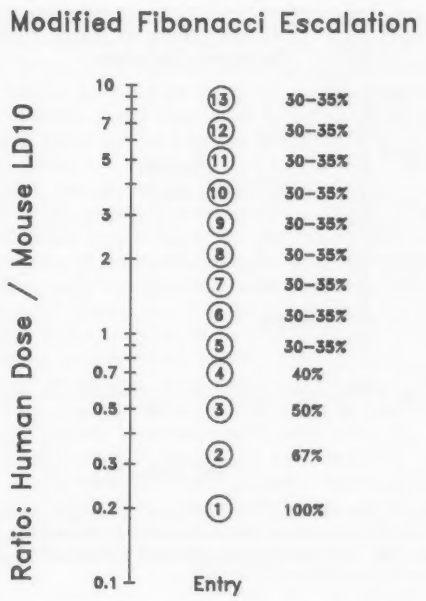


FIGURE 1.—Modified Fibonacci dose escalation scheme for phase I trials (semilogarithmic plot). The starting dose in humans is $\frac{1}{10}$ of the LD10 in mice. The first escalation step doubles the dose. Each subsequent escalation step is a decreasing % of the previous dose. Escalations continue at 30%-35% of previous dose until limiting toxicity (MTD) is reached (ref 10).

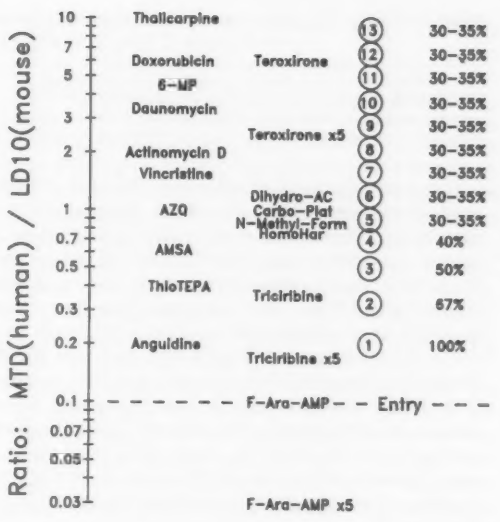


FIGURE 2.—Analysis of results for modified Fibonacci escalation when $\frac{1}{10}$ mouse LD10 is the entry dose. For these 17 drugs, the median MTD in humans was equal to the mouse LD10. The median No. of escalation steps to reach the MTD would be 5 or 6. For 5 of the drugs, $\geq 10$ escalations would be required. All drugs were given as single doses (× 1) or as 5 daily doses (× 5). Differences based upon schedule are noted. Data in the second column are from Grieshaber and Marsoni (ref 9). Data in the first column were collected from information in the literature or on file in the Toxicology and Investigational Drug Branches, Division of Cancer Treatment.

mouse LD10. The penalty is somewhat larger than might be realized. Since, as already reviewed, these drugs tend to end up at a human MTD about equal to the mouse LD10 (dashed line), figure 3 illustrates that an extra four to six escalation steps are required. Although "lost ground" is gained back quickly, the range of reduced escalation sizes is also entered rather early.

Again, it should be emphasized that there is no tailoring of this escalation process based upon pharmacology or other information. An alternative is to take advantage of pharmacologic data, and the next part of this paper describes how we are approaching this.

What are possible explanations for this variation in toxicity between mouse and man? Table 1 lists three possibilities: differences in drug metabolism/elimination/binding, exposure time differences, and target cell sensitivity differences.

Doxorubicin appears to be an example of metabolism/elimination differences. The MTD in man is fivefold greater than the LD10 in mice, on a mg/m$^2$ basis. Yet, as shown in figure 4, there is considerable agreement between blood levels measured at equitoxic doses. It appears that humans are more tolerant than mice due to a higher clearance (ml/min/m$^2$) for doxorubicin.

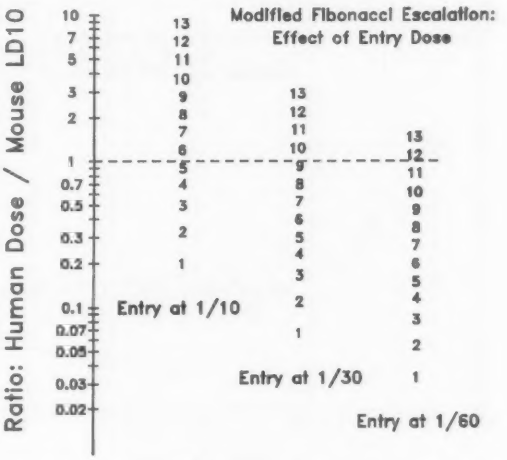


FIGURE 3.—Effect of starting dose on No. of escalations required to reach the MTD. For the median MTD, which equals the mouse LD10, 4 extra escalation steps are required when the entry dose is lowered from $\frac{1}{10}$ to $\frac{1}{30}$ of the mouse LD10. When the entry dose is further lowered to $\frac{1}{60}$ of the mouse LD10, 6 extra steps are required vs $\frac{1}{10}$ of the mouse LD10.

TABLE 1.—Potential explanations for variation in toxicity between mouse and man

| |
|---|
| 1. Species differences in drug metabolism/elimination/binding |
| 2. Schedule dependency due to exposure time differences |
| 3. Species differences in target cell sensitivity |

The second factor of possible importance is a difference in exposure times. For some drugs, there are threshold concentrations or time dependencies that are related to toxicity and/or mechanisms of action. For drugs with equal clearance values in mice and men (ml/min/m$^2$), Skipper and colleagues (4,6) have made the point that a bolus dose of equal mg/m$^2$ will have rather different time courses in mice and man (fig 5). If there is a threshold for action and a critical exposure time, where the threshold lies can give major differences in species response. For example, if the threshold in figure 5 is set at $10^{-6}$ M, there will be no effect in man. However, if the threshold is set at $10^{-7}$ M, the duration of effect will be much longer in man than in mice.

In a classic study by Quinn et al (12) 25 years ago, the threshold effect was first demonstrated for the barbiturate, hexobarbital. After a standard dose of 50-100 mg/kg was given to mice, rats, and rabbits, there was substantial variation in the drug effectiveness. The times to awakening were 12, 90, and 49 minutes in mice, rats, and rabbits, respectively. When the plasma pharmacokinetics of hexobarbital were investigated, it was found that the three species exhibit rather different elimination rates, or plasma half-times (fig 6). However, at the time of awakening, the plasma drug concentration was similar in all three species. Thus, this is an example of a species difference in drug effect which is determined by pharmacokinetic changes in exposure patterns.

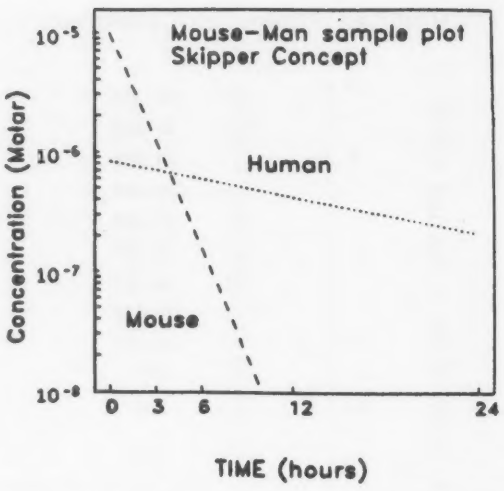


FIGURE 5.—Idealized plasma concentrations in mice and humans following equal bolus doses (mg/m$^2$). Assumes that volume of distribution (L/kg) and clearance (ml/min/m$^2$) are similar in both species. Adapted from Skipper et al (refs 4,6).

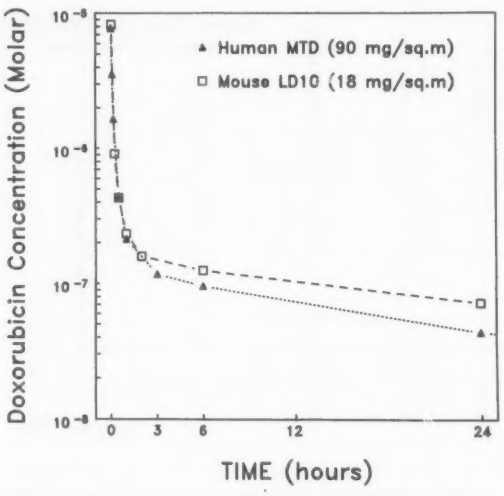


FIGURE 4.—Doxorubicin plasma concentrations in mice and humans at equitoxic doses. Human data scaled from 75-mg/m$^2$ dose (ref 11). Mouse data scaled from 75-mg/m$^2$ dose given iv to $CDF_1$ mice. Blood was collected by decapitation and analyzed by the methods of Greene et al (ref 11).

The first two reasons for the species variation in toxicology have been oriented towards plasma pharma-

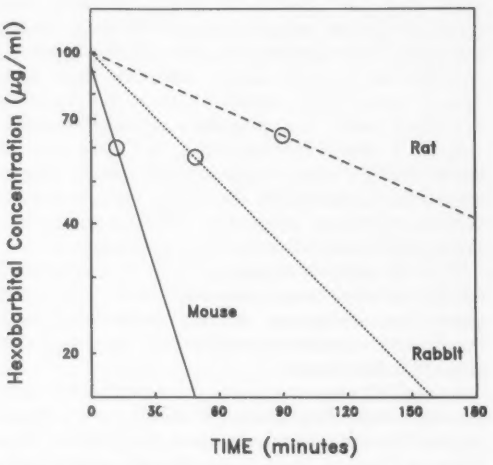


FIGURE 6.—Plasma disappearance of hexobarbital following 50-100-mg/kg doses given to mice, rats, and rabbits. Circles designate the concentration and time at which the animals awoke. Raw data from the study of Quinn et al (ref 12).

cokinetics. The third factor essentially covers all explanations which are not related to the delivery of the drug to the site of action. There can be differences at the cellular level which determine species sensitivity. For example, if the drug needs to be activated inside the cell, there may be a species difference in activation capabilities.

Fludarabine phosphate (F-ara-AMP) has been found to have the largest difference in dose (10- to 30-fold) between the mouse LD10 and the human MTD. This discrepancy is apparently an example of species differences in target cell sensitivity. The phosphate group on F-ara-AMP makes the drug readily soluble, but it is rapidly cleaved to F-ara-A in vivo. Within 5 minutes following administration, only the F-ara-A form can be detected in plasma. As shown in the plasma profiles following single doses of F-ara-AMP (fig 7), there is no obvious plasma pharmacokinetic explanation for the species difference in toxicity. In contrast to the situation for doxorubicin (where equitoxic doses produced similar plasma drug concentrations), it can be seen from these data that the plasma concentrations of the circulating species, F-ara-A, are considerably different.

## Specific Approach to the Integration of Pharmacology Into Phase I Testing

The final part of this paper discusses a specific approach to the integration of pharmacology into phase I testing. The focus is on species differences in elimination rates. Elimination rates determine the drug exposure or C × T. Initially, it is assumed that the C × T for the mouse LD10 and human MTD are equal, or similar. It is also assumed that threshold effects can be detected via schedule-dependency testing and target cell differences detected via in vitro screening. The proposal for C × T targeting will not apply to drugs with major differences in target cell sensitivity or schedule-dependent toxicity. We will return to these issues at the conclusion of this paper.

As outlined in table 2, the new procedure has five steps. The first four are data collection steps and are similar to what is currently done. Steps one and two require collection of pharmacokinetic and toxicologic data in mice; steps three and four are the collection of pharmacokinetic data at the human starting dose. The fifth step is a proposal to use the data collected in the first four steps to guide escalation strategy.

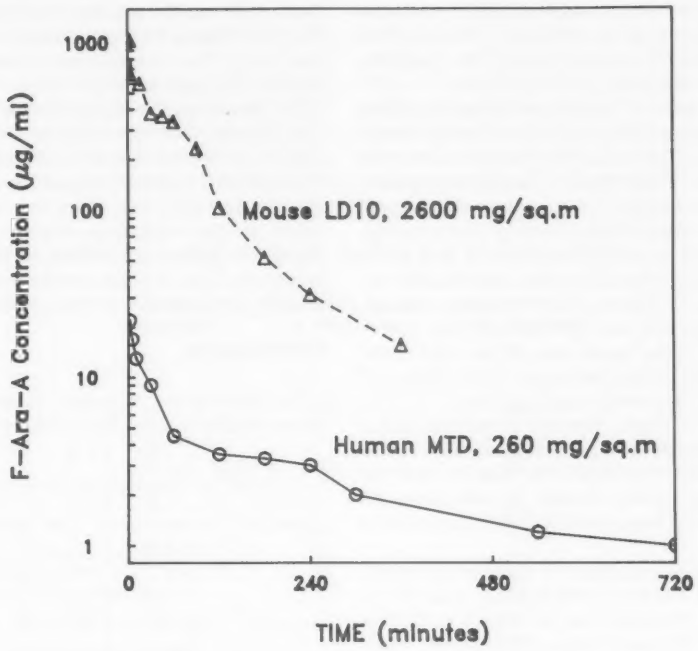


FIGURE 7.—F-ara-A plasma concentrations in mice and humans at equitoxic doses. Mouse data from Noker et al (ref 13). Human data from Malspeis (minutes of the Phase I Working Group meeting, Bethesda, MD, June 13–14, 1983).

TABLE 2.—Pharmacologically guided escalation of doses

Assume: C×T at mouse LD10 = C×T at human MTD

1. Determine mouse LD10 (current toxicology protocol).
2. Determine mouse C×T at LD10 (use preclinical pharmacology task orders).
3. Begin human testing at safe starting dose (1/10 LD10).
4. Measure human C×T at starting dose (pharmacokinetics is part of phase I contracts).
5. Choose escalation strategy based upon how close initial human C×T is to target C×T.

C×T information is listed for five anticancer drugs in table 3. For these five drugs, the C×T ratio is a useful predictor of the relative toxicity in mice and humans. For doxorubicin, as already discussed, the C×T ratio is far better than the dose ratio. For the other four drugs on this list, the C×T ratio was also a reasonable predictor, although it was about the same as the dose ratio. *The key difference is that the dose ratio is not known until the end of a trial, whereas the C×T ratio can be determined as soon as the trial begins.*

We will look at two examples of how such strategies might work. The initial C×T for amsacrine (AMSA) under this system would have been determined to be eightfold less than the target. Doxorubicin would have been further away, at 60-fold less than its target. Since the C×T for these two drugs are different distances from their target values, a different escalation procedure should be used for AMSA than for doxorubicin.

The details of potential escalation strategies depend upon a balance between the same forces of caution versus boldness which have led to empirical schemes such as the modified Fibonacci series. Table 4 lists two proposals. The underlying assumption is that pharmacology should be used to get the human plasma levels to the same range as in mice. Then, an empirical scheme can be used to finish the escalation procedure. The early steps are most important in terms of efficiency. Proposal A uses pharmacology to guide the first step by choosing a dose escalation factor equal to the square root of the ratio of the C×T at the mouse LD10 (ie, the target C×T) to the C×T at the entry dose. This could be called a "square root" or "geometric mean" method. Proposal B assumes that a doubling of the dose is the maximum permissible escalation. Therefore, doses continue to be doubled until the range of the target level is reached. In both cases, the modified Fibonacci scheme is used to finish the escalation process.

Figure 8 presents a comparison of these two proposals with the modified Fibonacci procedure for doxorubicin. Since doxorubicin enters far from its target, the potential savings are large (five to six steps, 50%). Figure 9 presents a similar comparison for AMSA. Since AMSA enters clinical testing closer to its target, the potential savings are lower (one to two steps).

Table 5 summarizes the potential savings in escalation steps for the five drugs listed in table 3. When the C×T ratio is an effective predictor of toxicity, these new procedures could reduce the number of escalation steps by about 20%–50%.

TABLE 3.—Mouse as a quantitative predictor of human toxicity. Comparison of dose ratio (mg/m$^2$ basis) and C×T ratio for human MTD to mouse LD10. Examples of drugs for which C×T appears to correlate with toxicity

| Drug | Dose ratio | C×T ratio |
|---|---|---|
| Doxorubicin | 5.0 | 0.8 |
| Diaziquone | 1.0 | 1.0 |
| Amsacrine | 0.8 | 1.3 |
| Pentostatin | 0.7 | 1.1 |
| Indicine *N*-oxide | 0.9 | 0.6 |

There are also some drugs for which the C×T ratio was not an effective predictor of toxicity (table 6). For PALA, both the dose ratio and the C×T ratio were overly conservative, ie, humans were threefold more tolerant than mice. Whenever humans are more tolerant than mice based upon a target cell sensitivity difference, a pharmacology-based dose escalation process would be safe, but not too much more efficient than an empirical dose escalation procedure.

A more serious case arises when humans are less tolerant than mice. Two such cases were found in our survey. F-ara-AMP, which has already been discussed, could have been troublesome for dose escalation based upon C×T ratios, except that no escalation is needed when the starting dose is already limiting.

Dihydroazacytidine was also a case where humans were less tolerant than mice, due to severe chest pain. As pointed out by Grieshaber and Marsoni (9), it is not easy to pick up some toxic effects in a mouse toxicology study. For dihydroazacytidine, there also would have been no safety problem with these strategies. Fortuitously, we would have landed very close to the MTD after one or two escalation steps. It is too narrow a line to follow intentionally, but it would have been acceptable for this drug.

## CONCLUSION

To summarize our experience thus far (tables 3 and 6), we are finding that the doses of more than one-half of the

TABLE 4.—Pharmacologically guided escalation schemes

| | |
|---|---|
| Proposal A: | Square root (or geometric mean) method |
| | First escalation step is equal to the square root of the ratio of the C×T at the mouse LD10 to the entry C×T. Subsequent steps follow Fibonacci scheme. |
| Proposal B: | Extended factors of 2 method |
| | Maximum escalation step size assumed to be factor of 2. Each escalation step is kept at factor of 2 until the human C×T is 40% of the C×T at mouse LD10. Then, step sizes are decreased according to Fibonacci scheme. |

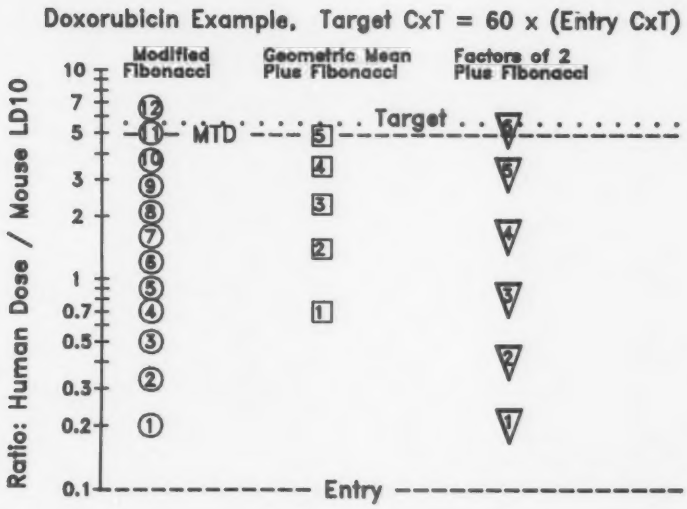


FIGURE 8.—Escalation strategies for doxorubicin.

drugs could be both safely and more efficiently escalated based upon C×T differences. In *none* of these eight examples would patients have had a higher risk in these proposed procedures than in the way the current system works.

The C×T concepts presented here can only be used if each new agent is screened for both schedule dependence and target cell sensitivity before entering clinical trials. Our most difficult challenge in predicting human toxicology on the basis of plasma concentrations will be to determine if a drug will be more like doxorubicin, or more like F-ara-AMP. There are some in vitro and in vivo tests which are probably feasible, and we are actively pursuing an evaluation in this area. In fact, such a screening test could also help us to avoid problems with the starting dose, so that it could be doubly beneficial.

If there is marked schedule dependency, then the continuous infusion schedule might be tested first. For continuous exposure to a drug, the steady-state drug concentration can replace C×T values. Steady-state concentra-

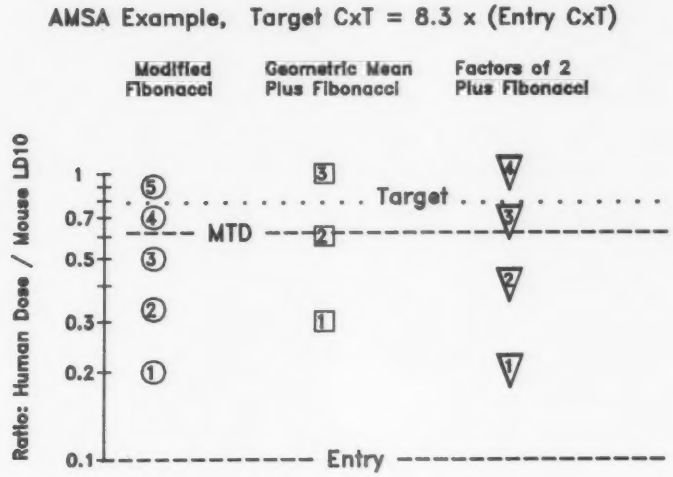


FIGURE 9.—Escalation strategies for AMSA.

TABLE 5.—Escalation steps required from entry dose (1/10 LD10) to MTD for various strategies

| | No. of escalation steps required | | |
|---|---|---|---|
| | Modified Fibonacci | Proposal A (square root) | Proposal B (extended 2's) |
| Doxorubicin | 11 | 5 | 6 |
| Diaziquone | 5-6* | 3 | 4 |
| AMSA | 4 | 2 | 3 |
| Pentostatin | 4 | 3 | 3 |
| Indicine *N*-oxide | 5 | 3 | 3 |

*Starting dose was actually 1/10 of MELD10, 9-10 Fibonacci steps required.

tion targets are more easily grasped and avoid the difficulties of exposure-time differences caused by pharmacokinetic factors. For both steady-state and C×T approaches, nonlinear pharmacokinetics and species differences in plasma binding must be considered.

Table 7 is an overview of the direction in which we are heading. Our projected plan is to complete a survey of about 20 drugs, and then to consider a formal proposal to change the general approach to phase I trials, perhaps with an intermediate step of a few pilot drug tests to gain further confidence in the new system. We are going back to reevaluate some established agents such as 5-azacitidine and melphalan, which are known to have dose differences between mouse and man, to find out if toxicity correlates with C×T. A number of new drugs have just begun, or will shortly enter, phase I testing. We have set up the "pipeline" to follow these agents.

We look forward to seeing the fruition of these efforts as new drugs enter phase I testing with sufficient pharmacologic support to improve the overall effort.

TABLE 6.—Mouse as a quantitative predictor of human toxicity. Comparison of dose ratio (mg/m$^2$ basis) and C×T ratio for human MTD to mouse LD10. Examples of drugs for which C×T does not appear to correlate with toxicity

| Drug | Dose ratio | C×T ratio |
|---|---|---|
| PALA | 2.8 | 3.3 |
| F-ara-AMP | 0.03-0.1 | 0.1 |
| Dihydroazacytidine | 1.2 | 0.3 |

TABLE 7.—Mouse as a quantitative predictor of human toxicity. Comparison of dose ratio (mg/m$^2$ basis) and C×T ratio for human MTD to mouse LD10

| Drug | Dose ratio | C×T ratio |
|---|---|---|
| 5-Azacitidine | 6.0 | * |
| Teroxirone | 6.0 | * |
| Doxorubicin | 5.0 | 0.8 |
| PALA | 2.8 | 3.3 |
| Dihydroazacytidine | 1.2 | 0.3 |
| Diaziquone | 1.0 | 1.0 |
| Indicine *N*-oxide | 0.9 | 0.6 |
| AMSA | 0.8 | 1.3 |
| Pentostatin | 0.7 | 1.1 |
| Melphalan | 0.4 | * |
| Thiotepa | 0.4 | * |
| F-ara-AMP | 0.03-0.1 | 0.1 |
| Tiazofurin | Phase I trials in progress or beginning soon | |
| Phyllanthoside | | |
| Pibenzimol | | |
| Caracemide | | |
| Menogaril | | |
| HMBA | | |
| Trimetrexate | | |
| BIDA | | |

*Pharmacokinetic studies in progress.

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# Body Burden of Cancer in Relationship to Therapeutic Outcome: Consideration of Preclinical Evidence

**Daniel P. Griswold, Jr**[1,*]

"Surgery or radiation therapy fails to cure clinically evident human cancer, in the main, if the disease is systemic (has metastasized) when first recognized, because neither modality can effectively remove or kill distant and/or unrecognized metastases. Starting drug treatment of a tumor when it is first clinically recognized, or when it re-presents following noncurative surgical or radiological treatment, fails to cure over 90% of cancer in man because *the body burden of tumor cells exceeds the tumor cell kill potential* of nearly all of the most effective drugs or drug combinations against most tumors" (emphasis added) (1). That quotation is from a paper written by Frank M. Schabel, Jr, in 1977 on the "Rationale for Adjuvant Chemotherapy." Now, 7 years later, it is perhaps appropriate to reexamine the basis for the accepted view that small tumor burdens are more responsive than larger burdens to chemotherapy.

Nearly 30 years ago, Goldin et al (2) showed that a relationship existed between the size of a leukemic cell inoculation and the amount of drug dosage necessary to obtain similar "curative" effects. This important observation on the dose-responsive nature of tumor cell populations was followed by a report from Martin and Fugmann (3) that showed the existence of a similar relationship in a solid tumor. In their studies, they used the murine adenocarcinoma 755 (Ca 755) and 6-mercaptopurine. Treatment of sc implanted tumor-bearing mice was initiated in different groups on Day 1, 8, or 15, allowing the tumors in each group to be successively larger. Treatment was continued for 2 weeks, and the mice were observed for an additional 2 weeks after the cessation of treatment. Tumor "cures" at that time were 57%, 26%, and 0%, respectively. Although it is now recognized that those were probably not "cures," the inhibition of tumor growth was clearly related to the size of the tumors at the time treatment was initiated. In another series of experiments reported in the same paper, they showed that partial surgical excision of 15-day-old tumors followed by treatment with 6-mercaptopurine resulted again in 57% tumor-free mice at the time of evaluation, while surgery or chemotherapy alone was ineffective.

Thus, it was shown prior to 1960 not only that there is a correlation between the size of chemotherapeutic dose and the response obtained in tumor cell populations, but that "small" leukemic and solid tumor cell populations are more responsive than larger ones. As more effective cytotoxic anticancer drugs and improved biological techniques became available, those observations were conclusively confirmed (4-7). Examples are shown in tables 1 and 2.

The question then became: Why should a small tumor burden be more responsive (curable) than a large burden? Is it simply a matter of an overwhelming number of cells, or is tumor size only indirectly responsible? The latter would seem to be the case for several reasons.

## Differential Cytotoxicity

In 1964, Skipper et al (4), using an experimental mouse leukemia, provided the basis for planning chemotherapy trials in a quantitative sense. Among other important observations, they showed that the killing by many cytotoxic drugs of sensitive tumor cells follows a pseudo first-order kinetic rate. That is to say that the fraction, not the absolute number, of drug-sensitive cells killed by a given dose of drug is the same, regardless of the size of the cell population at the time treatment is initiated. Thus, a drug treatment that would reduce $10^4$ sensitive cells to $10^1$ would also reduce $10^9$ to $10^6$. Unfortunately, the concept was often misunderstood simply because of failure by some to recognize that it applies only to drug-sensitive cells. Tumor cells may be insensitive for a variety of reasons, including, but not limited to, specific biochemical resistance, cell cycle characteristics, and pharmacologic and/or anatomic barriers. [Skipper (8) has more recently addressed this subject in much greater detail.]

Nevertheless, the concept offered the cautious hope of "cure," even of large tumor burdens. Mathematical models were derived to show the interrelationship between the net tumor cell kill obtainable and the cell kill from a single dose or course of drug, the number of doses or courses, the time interval between those doses or courses, and the doubling time of the tumor cell population that survives each dose or course. Based on these

[1]Southern Research Institute, Birmingham, AL.

[*]*Reprint requests to:* Dr. Daniel P. Griswold, Jr, Southern Research Institute, PO Box 55305, Birmingham, AL 35255-5305.

early models, it was theorized that tumors even in advanced stages could be totally eradicated by proper choice of drug and treatment schedule (*a*) *if* all the cells were in a sensitive state and (*b*) *if* treatment could be given over a sufficiently long time period. Obviously (*a*) does not occur in vivo and (*b*) becomes both impractical and impossible.

Most available effective anticancer agents are cytotoxic, deriving their benefit from killing cancer cells (or rendering them nonproliferative). They also kill normal tissue cells, particularly rapidly dividing cell populations such as hematopoietic and intestinal epithelial cells. Thus, the numbers of cancer cells that can be eradicated by chemotherapy are limited by the cytotoxic specificity with each single dose or single course and by the cumulative normal tissue toxicity of the total dosage of the drug(s) employed. An analysis has been made of the therapeutic indices [TI = LD10 (mg/kg)/minimum effective dose (mg/kg)] and LD90/LD10 ratios at the optimal schedule for each of 12 clinically effective anticancer drugs that were used to treat mice with the L1210 leukemia (9). For those representative 12 drugs, the median TI value was 3.2 and the median LD90/LD10 ratio was 2.0. The results are graphically depicted in figure 1 and clearly show the very narrow range of drug dosages between those that provide minimal antitumor activity and the dosages that are lethally toxic. Steep dose-response curves are characteristic of cytotoxic anticancer drugs and severely restrict the tumor cell kill that can be obtained from any single dose or course of chemotherapy. To further compound this problem of cytotoxic specificity, it has been shown that recovering hematopoietic and intestinal epithelial stem cells become increasingly sensitive to successive treatments because of an induced high-proliferative rate (10-12). Thus, without proper treatment scheduling, the differential cytotoxicity will be reduced or even negated (12).

Cumulative toxicity, particularly for certain cases of anticancer drugs, is also a well-recognized problem. The cardiomyopathy induced by doxorubicin is one example. Delayed toxic effects from certain of the nitrosoureas are another. (*See* ref 13 for a review of the toxic effects of most clinically available anticancer drugs.) Thus, it is clear that curative potential is inversely related to size of a tumor cell population, at least in part, because the toxicity of available drugs limits the individual and total dosage that may be used safely.

TABLE 1.—Response of ip L1210 leukemia in mice to fractions of single-dose LD10 of cyclophosphamide*

| Single dose | | No. of L1210 cells | % ILS† excluding cures | % cures |
|---|---|---|---|---|
| Mg/kg | Fraction of LD10 | | | |
| 300 | 1.0 | $10^7$ | 200 | 40 |
| | | $10^6$ | — | 80 |
| 230 | 0.77 | $10^5$ | 70 | 82 |
| 173 | 0.58 | $10^5$ | 80 | 42 |
| 115 | 0.38 | $10^5$ | 60 | 20 |
| 58 | 0.19 | $10^5$ | 21 | 5 |
| 25 | 0.08 | $10^5$ | 10 | 0 |
| 12.5 | 0.04 | $10^5$ | 0 | 0 |

**Modified from:* Griswold DP, Jr, Corbett TH, and Schabel FM, Jr. Pharmacology and development of new therapeutic agents. *In* Gastrointestinal Cancer (Stroehlein JR, and Romsdahl MM, eds). New York, Raven Press, 1981, pp 443-455. Used with permission.

†ILS = increase in life span.

## Tumor Cell Population Kinetics

During the decade of the 1960s and early 1970s, there was a great interest in tumor cell population kinetics and the manner in which tumor growth characteristics might affect therapeutic outcome. The volume increase with age of a number of experimental transplantable tumors could be described by a Gompertzian equation which showed, to varying degrees depending on the tumor, that growth became asymptotic in the later stages (14). Backward extrapolation of such growth curves suggested an increased rate of cell proliferation or fraction of proliferating cells with decreasing population size and offered hope that those smaller cell populations would be more susceptible to chemotherapy, especially with antimetabolites. As knowledge of cell population kinetics increased, it was recognized that the rate of tumor volume growth was a function of cell cycle time, growth fraction, and cell loss (15-17). When these kinetic parameters were examined in different stages of tumor growth, it was indeed observed with certain murine tumors that with increasing tumor volume there was a concomitant increased cell cycle time and decreased proliferative fraction (14). Again, by inference, such observations were taken to suggest that very small tumors might have short cell cycle times and high growth fractions—hopefully an exploitable chemotherapeutic situation.

In experimental systems, a therapeutic advantage has been shown to exist in "small" as opposed to "large" tumor cell populations. DeWys (18) showed that the fractional tumor cell kill in the Lewis lung tumor was greater

TABLE 2.—Effect of tumor staging on curability of subcutaneous B16 melanoma*

| Tumor size at therapy† (No. of cells) | % mice cured |
|---|---|
| $\geq 10^9$ | 0‡ |
| Fragment or $10^7$ | 4 |
| $10^5$ | 18 |
| $10^4$ | 33 |
| $10^3$§ | 70 |

**Modified from* ref 7.

†Mice were treated with a single dose of semustine (40 mg/kg). Pooled data from 6 experiments, 10 mice/group in each experiment.

‡13% responders.

§Normalized value based on 10% no-takes in the untreated controls and 78% "cures" in the drug-treated groups. There were no no-takes in any of the other control groups.

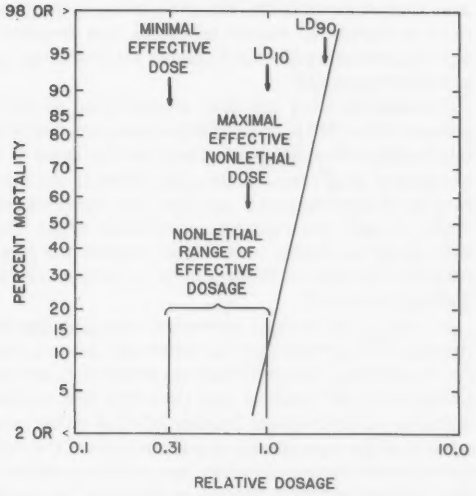


FIGURE 1.—Dosage-mortality curve derived from the medium LD10 and LD90 values of 12 clinically used anticancer agents in mice, and the minimal effective and maximum tolerated doses of the same agents when used to treat L1210 leukemia in mice. *Taken from:* Griswold DP, Jr, Corbett TH, and Schabel FM, Jr. Pharmacology and development of new therapeutic agents. *In* Gastrointestinal Cancer (Stroehlein JR, and Romsdahl MM, eds). New York, Raven Press, 1981, pp 443-455. Used with permission.

both in leg tumors and lung metastases when cyclophosphamide was given early (Day 8 after tumor implant) as compared to results obtained from identical treatment given on Day 15 or 21. That greater cell kill was presumed to have occurred because the smaller tumor cell populations may have been in a more rapid state of proliferation. Two other studies suggested that reduction of a tumor cell population by chemotherapy resulted in a shorter cell cycle time and/or larger growth fraction of surviving cells and thereby allowed enhanced tumor cell killing by subsequent drug treatment (19,20). Those results, however, may have been unique, since both tumors were unusually sensitive to the agents employed and marked tumor cell kill could be obtained from small fractions of the maximum tolerated doses.

In other studies using experimental systems, attempts have been made to time chemotherapy to coincide with postsurgical recovery of residual tumor or with proliferative recovery after an initial course of chemotherapy (21,22). More importantly, it was shown that tumor cell proliferative recovery could be controlled by dexamethasone treatment so that the timing of chemotherapy could be more appropriately aligned with a high-proliferative rate (22,23).

Whether or not any of these findings in experimental systems will ever have clinical relevance remains to be seen. It may be that the observed kinetic changes that result from surgical or chemotherapeutic perturbation are of insufficient magnitude to make any marked difference in the treatment of human cancer. There are several excellent reviews of this subject (24-26).

## Metastasis and Surgical-Adjuvant Chemotherapy

The earliest notions about surgical-adjuvant chemotherapy were based on the assumptions by some that chemotherapy might be used to destroy those malignant cells released into the circulation at the time of surgical ablation of the primary tumor (27). It was subsequently shown that a large percentage of patients have metastatic disease at the time of or prior to detection of the primary tumor. In a large patient series it was clearly shown that a trend exists toward a direct relationship between primary tumor size at the time of surgery and the existence of metastatic disease (28). These same data, however, show that there is great variability among individual patients. The tumor size/metastasis relationship was recently reported in another large series (29).

This same correlation was demonstrated in metastatic animal tumor systems (30) and led to the design of preclinical surgical-adjuvant trials in which the tumors were deliberately "staged" at different sizes in order to determine the effect of chemotherapy on metastatic disease. The results of those studies showed that surgical cure was inversely proportional to the size of the primary tumor at the time of surgery and that cure rates were improved by chemotherapy following surgery (7,31). *See* figures 2 and 3 for examples.

Again, based on preclinical experiments with animal tumor systems, the evidence pointed towards a favorable therapeutic outcome with the smaller tumor cell populations. The results, however, were not always as clear-cut as hoped, and failures occurred even in some of the carefully staged experimental systems. It is now recognized that failure to eradicate very small tumors may result from any one of several possibilities. Just as in women with cancer of the breast, the time of metastasis in relation to the size of the primary is a variable phenomenon in mice with sc or im implanted tumors. When mice bearing identically sized im implants of Lewis lung tumor were randomized into five groups, had their tumor-bearing legs surgically removed at the same time, and were observed for death, the mortality from metastatic tumor growth ranged from 30% to 90% in the different groups (32). Skipper and Schabel (33) have shown that within a single group of mice with similar sized primary tumors, there is a wide distribution of individual mice with varying metastatic burdens. Based on available evidence, those with the largest metastatic burdens may be beyond the "curable" potential of adjuvant chemotherapy.

Additionally, metastasis to anatomically privileged sites, such as brain, creates an additional therapeutic problem. The probability of that event may increase with increasing primary tumor age and size (34). Fidler et al

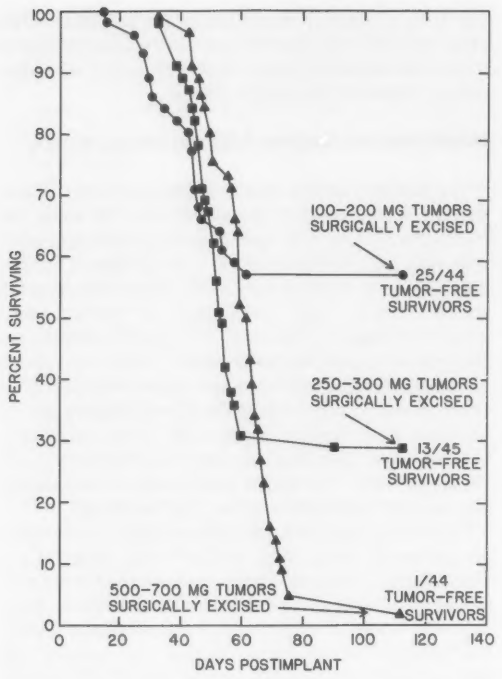


FIGURE 2.—Survival of mice bearing sc C3H mammary adenocarcinoma after surgical excision of the sc tumor at various stages of growth. *Taken from* ref 7.

(35) and Martin (27) have written extensive reviews on metastasis and adjuvant chemotherapy.

### Drug Resistance

It is now clearly recognized that the selection and overgrowth of specifically drug-resistant tumor cells are associated with increasing tumor cell population size and represent a major obstacle to curative chemotherapy. More than 60 years ago, two mechanisms were argued as being responsible for the development of drug resistance. One suggested that resistance resulted from exposure to the agent in question; the other held that resistance was random and spontaneous occurring in absence of the agent. Subsequently, the very important work of Luria and Delbruck (36) and, later, Law (37) showed that resistant cells may occur randomly and spontaneously. This is of primary importance in recognizing that at the time of clinical diagnosis, prior to any treatment, a tumor may contain drug-resistant subpopulations of cells (38). Although mutation to resistance is a random event, it is random within limitations, eg, the growth kinetics of the particular tumor. Schabel et al (38) have indicated from their studies of a large variety of drug-resistant leukemias that, in general, the rate of mutation to drug resistance is highest to mitotic inhibitors, less frequent to antimetabolites, and lowest to highly reactive drugs such as alkylating agents.

Considerable work has been accomplished at the experimental level to provide a better understanding of the relationship of tumor age or size to the likelihood of the presence of drug-resistant cells, the effect of their presence on chemotherapeutic outcome, and the cross-resistance patterns that exist among different drugs. There have been two recent reviews that discuss the subject from the viewpoint of the preclinical experimentalist and the clinician (39,40).

In view of the present discussion regarding the relationship of drug resistance and tumor size, several points are noteworthy. The mathematical model of Goldie and Coldman (41,42) "predicts that there will be a variation in the size of the resistant fraction (of cells) within tumor colonies of the same size for any given value of the mutation rate, and furthermore that there will be a sudden decline in the expectation for cure as the (total) cell number increases." Thus, there is a direct relationship between size of the tumor burden and the presence of resistant cell populations. If the growth kinetics of the sensitive and resistant cell subpopulations are similar, then the resistant fraction will remain the smaller until the resistant fraction is selected, by reduction of the sensitive fraction, allowing the resistant cells to overgrow the sensitive ones. (Depending on the mutation frequency and the growth kinetics of the sensitive and resistant populations, it is possible that larger tumors may have higher ratios of resistant to sensitive fractions.) This has been done deliberately to develop single and double drug-resistant experimental tumor cell lines for almost all known useful cytotoxic agents. It is done inadvertently in the treatment of animal and human tumors. The presence of a resistant subpopulation, however, may go unnoticed for some time. Skipper (43), using various ratios of drug-sensitive and drug-resistant murine leukemic cells, mixed in a common inoculum, has shown that clinical failure will not be observed until the resistant subpopulation reaches about 50% of the total. Thus, clinically observed tumor responses reflect the eradication only of the drug-sensitive fraction of cells. Recurrence in the face of continued undiminished treatment usually reflects overgrowth of that fraction that is resistant to the drug(s) being used.

## CONCLUSION

The purpose here was to consider from a preclinical point of view how tumor burden might affect the results of therapy, particularly chemotherapy. Four possible factors (not that other reasons do not exist) have been considered:

1. The limitations imposed by toxicity to normal tissues, lack of sufficient differential toxicity, and cumula-

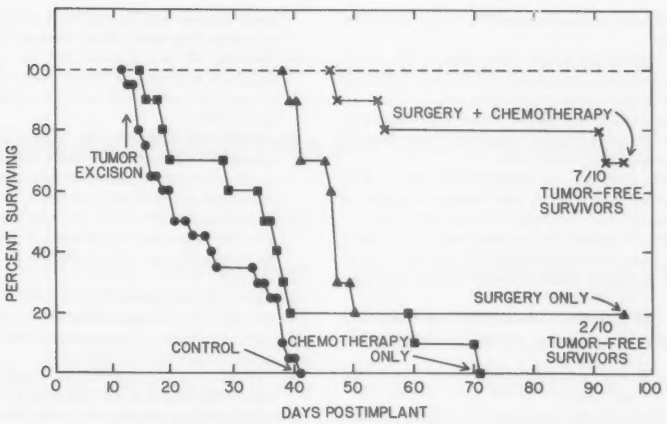


FIGURE 3.—Survival of mice implanted sc with B16 melanoma (trocar fragments) after surgical excision alone of the primary sc tumor (tumor size at time of surgery = 0.9–1.1 g), treatment with semustine alone (36 mg/kg, single dose), or surgical excision with chemotherapy 1 day later. *Taken from* ref 7.

tive toxicity—limiting the drug exposure that can safely be attained;

2. The influence of cell population kinetics, particularly so with drugs to which tumor cells exhibit concentration independence;

3. The risk of metastasis with increasing tumor size or age—especially to anatomic/pharmacologic sanctuaries; and

4. The direct relationship of the presence of drug-resistant cells and size of the tumor burden.

Unlike the situation with many antibacterial antibiotics that may be given at high doses and for long durations with a wide margin of safety, the presently known cytotoxic anticancer agents must be given with much greater care. The limitations of their effectiveness (for the reasons discussed above), at concentrations and durations that may be safely employed, dictate the need for improved diagnostic tools, careful disease staging, and the most appropriate treatment scheduling.

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# Adjuvant Chemotherapy—Clinical Trials and Laboratory Models

**Robert E. Wittes**[1,2,*]

Without doubt, the most important clinical hypothesis in cancer over the past two decades has been that chemotherapy regimens, which are at best palliative in the setting of advanced cancer, can increase the cure rate of standard therapy when used very early in treatment. This concept was first subjected to formal clinical testing in the 1950s (1). Clinical trials were motivated and reinforced by clear demonstrations in laboratory models that for many tumors in a variety of hosts, chemotherapy was vastly more effective after surgical cytoreduction than without such pretreatment (2-4). By the early 1970s, enthusiasm for combination chemotherapy, whose potential had already been amply demonstrated in the hematologic malignancies, was coupled with an increasing emphasis on the incorporation of chemotherapy into initial treatment strategies. At the same time, intensive efforts at the Southern Research Institute and elsewhere solidified the conceptual basis for the therapy of micrometastases. These developments in the clinic and the laboratory stimulated a very large number of adjuvant trials across the whole spectrum of human cancer.

The results of this effort have been mixed. On the one hand, the experience shows that the early use of chemotherapy can increase disease-free survival and, in many instances, survival after primary treatment in several kinds of cancers, including Wilms' tumor (5), rhabdomyosarcoma (6), osteosarcoma (7,8), Ewing's sarcoma (9), Hodgkin's disease (10), ovarian carcinoma (11), and breast cancer (12,13), at least for certain patient subsets in each disease category.

In an admittedly larger number of diseases, however, including many of the common epithelial cancers of adulthood, efforts to date have not yielded positive results. The fact that adjuvant chemotherapy programs have not produced a quantitatively large improvement in survival for many neoplasms has led in certain quarters to a disenchantment with the whole concept. Some have argued that the therapeutic index of currently available cytotoxic agents is simply too low to expect much of this approach in human beings.

Before deciding that a concept has failed, however, we should make certain that it has been adequately tested. Rather than indulge in polemics about whether adjuvant chemotherapy "works" or not, we should ask critically what the clinical experiments have actually taught us. To what extent have they reproduced the conditions of the laboratory models in which success with this approach is so dramatic? If, in fact, the clinical trials have not been reasonable replicas of the models, why have we failed to do the right experiments? Have we not understood how to translate experiments in mice into a clinical setting? Have we been too cavalier about the necessity of doing so? Or are the conditions of the murine experiments, for one reason or another, simply impossible to translate into the clinic more faithfully than we have?

## Murine Cancers as Models of Human Cancer

Experimental chemotherapists have accumulated a large body of data showing persuasively that chemotherapy is frequently curative against low tumor burdens (table 1). Most of these experiments have been performed in transplantable tumors of mice. The extent to which these tumors really constitute adequate models for spontaneous autochthonous human cancer is certainly an appropriate concern. Indeed, as results in the clinic appear to diverge from what can be achieved in the models, the reaction of disgusted clinicians is often that the models are irrelevant.

Transplantable murine tumors and human cancers share many important behavioral characteristics. As Griswold (14) has noted, murine tumors metastasize, exhibit a reasonable growth rate (considering the lifetime of the host), and show considerable heterogeneity in response to treatment. In fact, the response of a given murine tumor to a specific chemotherapeutic agent can vary remarkably from line to line with very similar passage histories (15).

As shown in table 2, advanced murine tumors, like their human counterparts, are generally incurable with chemotherapy (15). Exposure of initially sensitive murine tumors to single agents usually results in an initial re-

[1]Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

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*Reprint requests to:* Robert E. Wittes, MD, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Landow Bldg, Rm 4A22, National Institutes of Health, Bethesda, MD 20892.

sponse, followed by the rapid emergence of drug resistance (16). In at least two obvious respects, however, differences between transplantable tumors and clinical cancer may pose significant problems in interpretation for the clinician. First, the way in which most adjuvant experiments are performed in murine models (transplant tumor to a new host, allow time for growth, resect the "primary" tumor, wait a variable period of time, then treat the residual micrometastases with chemotherapy) is at best a rather stylized recreation of the early natural history of spontaneous autochthonous cancer. In the laboratory, the experimentalist defines accurately a zero time for the process of metastasis. Transplantable tumors are sufficiently well characterized so that the approximate body burden of tumor and its anatomic localization may be known in quite considerable detail, at least in a statistical sense.

The clinician is not so fortunate. When adjuvant treatment begins following resection, the physician knows only that the patient has a certain probability of relapse and that the staging radiographs and scans have ruled out the presence of large metastatic tumor masses in certain locations. He does not really know whether he is treating micrometastases or occult gross disease that has escaped detection by a few crude screening tests. Since, for most cancers, chemotherapy regimens that cannot cure obvious gross disease probably cannot cure occult gross disease either, simple staging errors, unavoidable with current technology, may be a major cause of apparent therapeutic failure. These kinds of failures really do not reflect adversely on the potential of treatment to eradicate micrometastases.

Staging errors are probably of some practical importance. In a series of autopsied lung cancer patients dying of causes other than malignancy within 30 days of diagnosis (17), the incidence of distant metastases undetected by preoperative staging in the non-small cell lung cancer group was 24%. Shields (18) has estimated that 9%–27% of non-small cell lung cancer patients with T1N0 and T1N1 disease and 12.5%–40% of those with T2N0 and T2N1 disease harbor occult distant macrometastases.

Second, transplantable murine tumors seem significantly more sensitive to existing chemotherapeutic agents than human cancers are (15). This is probably not surprising, since nearly all anticancer drugs were initially brought to clinical trial on the basis of activity in murine systems. The basis for this difference is not entirely clear; the faster growth rates and larger growth fractions of many experimental tumors may make them more vulnerable targets (19–21). Table 2 shows the response rates, defined in the conventional clinical fashion, of a number of single agents against several advanced murine tumors that are commonly used as adjuvant models. At least by the standards of the clinical oncologist,

Table 1. Animal Tumor Systems in Which Adjuvant Chemotherapy Increases the Cure Rate (data from References 2, 3, 4, 16, 17, 54)

| Tumor | Host | Treatment |
|---|---|---|
| Mammary 755 Adenocarcinoma | Mouse | 6-MP<br>Puromycin aminonucleoside<br>6-aminonicotinamide |
| RC adenocarcinoma | Mouse | 6-MP |
| Sarcoma 180 | Mouse | 6-MP |
| Walker 256 Mammary adenocarcinoma | Rat | Thio-TEPA |
| S-91 melanoma | Mouse | Thio-TEPA |
| Lewis Lung carcinoma | Mouse | Cyclophosphamide<br>Methyl-CCNU |
| B16 melanoma | Mouse | Methyl-CCNU |
| Colon 26 | Mouse | Methyl-CCNU |
| C3H mammary/16 | Mouse | Doxorubicin<br>Melphalan |
| Spontaneous mammary adenocarcinoma | Mouse | Uracil mustard<br>Combination chemotherapy |

response rates, and in some cases complete response rates, are very high. In general, these high response rates seem to translate into survival advantages for the treated population compared to untreated controls (% ILS). Since anticancer agents are more effective against gross murine tumors than against most gross human cancers, it might not be unreasonable to expect greater efficacy against early murine disease than against early human disease as well.

Although by far most of the studies in this area have utilized transplantable tumors, a few have focused on spontaneous tumor models. Martin's (22) early studies of chemotherapy in the spontaneous mammary adenocarcinoma of $CD8F_1$ mice showed that tumor "cure" (defined as histologic absence of tumor after treatment) is greatly enhanced by the use of chemotherapy immediately after surgery. Later experiments utilizing combination chemotherapy in which animals were followed to death confirmed the striking efficacy of postoperative treatment in increasing cure rates (23).

Because no animal tumor model is a precise reflection of clinical disease, we should only attempt to draw broad lessons from studies of the relationship of treatment results to the various parameters of interest. According to Schabel (24,25), these are that a maximally effective regimen should be given as intensively as possible, as early after removal of the bulk of tumor as possible, to a host that has the lowest possible body burden of tumor. In many cases, drug combinations are substantially more effective than single agents (23,26).

Let us now examine in detail how these lessons from the laboratory have been moved to the clinic.

## Dose

Dose, or dose intensity (defined as dose per unit time during the treatment period), is probably the most important variable that determines the efficacy of experimental treatment programs in the laboratory. In a wide variety of systems, effectiveness increases with increasing dose to the limits of host tolerance. As Schabel et al (15) have stated: "The dependence on maximum dosing for greatest tumor cell kill and optimum therapeutic effect is probably the most consistent principle in cancer chemotherapy that we predictably observe with essentially all drugs and certainly with all murine models."

Clinicians should be aware that to an experimental chemotherapist the limits of host tolerance are defined by death from drug toxicity. The effectiveness of chemotherapy continues to increase with dose until the tumor-bearing animals begin to die from the adverse effects of the drugs. The dose dependence of antitumor efficacy is

Table 2: Response Rates and Cure Rates of Several Transplantable Murine Tumors When Treated With Single Drugs in Advanced Stages (Data Adapted from Reference 15)

| | Ridgway Osteogenic Sarcoma | | Lewis Lung | C3H 13/C Mammary | Colon 38 | B16 Melanoma |
|---|---|---|---|---|---|---|
| Size of primary tumor mass (gm) | ≤1 | ≥2 | 0.4 | 0.1 - 0.6 | 0.1 - 0.6 | 0.5 |
| Actinomycin D | 91,84,75(S) | 82,19,2(S) | - | - | - | - |
| Adriamycin | 82,68,3(S) | 10,0,0(S) | - | 49,21,4(R) | 10,10,2(R) | - |
| Anguidine | - | - | - | - | 63,27,19(R) | - |
| Ara-C | 79,63,1(R) | 72,17,0(R) | - | - | - | - |
| Ara-C (Palm O) | - | - | - | - | 25,25,5(R) | - |
| Bleomycin | 20,0,0(R) | - | - | 10,10,0(R) | - | - |
| BCNU | - | - | 5,0,0(S) | 40,0,0(S)* | - | - |
| CCNU | - | - | 15,7,3(S) | - | - | - |
| Cisplatin | 90,85,5(R) | 65,20,0(R) | - | - | - | - |
| Cyclophosphamide | 96,96,37(S) | 94,18,0(S) | 60,24,22(S) | 50,10,0(S) | 4,2,0(R) | 13,2,1(S) |
| | 100,100,60(R) | 92,65,29(R) | 60,20,10(R) | 29,11,1(R) | - | 19,0,0(R) |
| Dianhydrogalactitol | - | - | - | 30,10,0(R) | 20,10,10(R) | - |
| Daunorubicin | 75,25,0(R) | - | - | - | - | - |
| Dibromodulcitol | - | - | - | - | 3,3,0(R) | - |
| FU | 85,55,0(R) | 50,5,0(R) | - | 28,12,2(R) | 26,8,1(R) | - |
| MeCCNU | - | - | 38,16,8(S) | - | 60,20,0(R) | 8,0,0(S) |
| | - | - | 53,25,8(R) | - | - | 8,3,1(R) |
| 6-MP | 50,70,0(R) | 50,0,0(R) | - | - | - | - |
| Melphalan | - | 97,23,7(S) | - | 40,40,0(R) | - | - |
| | - | 93,80,20(R) | - | - | - | - |
| Mitomycin | 15,0,0(S) | - | - | 40,25,0(R) | 3,3,0(R) | - |
| PALA | 30,2,2(R) | - | - | - | - | - |
| Procarbazine | - | - | - | - | - | - |
| Vinblastine | - | - | - | 27,13,3(R) | - | - |
| Vincristine | 90,69,3(R) | 77,3,0(R) | - | 20,8,0(R) | - | - |
| VP-16 | 50,0,0(S) | - | 20,0,0(R) | - | - | - |
| | 60,50,0(R) | - | - | - | - | - |

Footnote to Table: The three figures in each entry refer to complete plus partial response rate, complete response rate, and cure rates for each disease-drug combination. S = single dose schedule; R = Repeated dose schedule.

seen in laboratory models of adjuvant therapy (14,24-28) just as in the single-modality therapy of early or late murine disease (15). For example, in the adjuvant treatment of C3H mammary tumors with CCNU-cis and cyclophosphamide, a 33% decrease in dose produces a nearly 40% reduction in cure rate and a clear decrease in the median survival time of dying animals (25). Moreover, in at least one system the dose-response relationship may be steeper in the adjuvant setting than in the absence of surgical cytoreduction (29).

Within the past few years, the importance of dose in the treatment of human neoplasia has been emphasized repeatedly (30-32). In early clinical trials the assumption is usually that the "maximum tolerated dose" (MTD), defined as the highest dose producing acceptable degrees of toxicity in phase I studies, is at or near the appropriate level for wider exploration in phase II studies. For drugs having myelosuppression as the dose-limiting toxicity, the evolution of techniques for bone marrow transplantation has permitted exploration of dose ranges well above the conventional MTD. In general, such studies have confirmed that antitumor effect increases with increasing dose over a wide range of doses (33-35).

In routine clinical practice, however, despite the widely acknowledged steep dose-response curve of chemotherapeutic agents, the actual administered doses of many commonly used combinations are often quite conservative. Unlike their laboratory colleagues, clinical oncologists have obviously never used the number of toxic deaths as an indication of how much drug they can give. In phase I studies involving systematic escalation of dose, clinicians use the incidence of major but reversible toxic effects as the principal endpoint. Once a regimen enters the arena of widespread clinical testing, to say nothing of standard practice, there is a tendency to shave doses to assure the patient as much freedom from troublesome toxic effects as possible. This is particularly likely to occur when some trials have suggested that dose may not be such a critical variable after all (36).

Two additional peculiarities of the adjuvant setting tend to encourage dose reduction. First, since a proportion of patients treated with adjuvant chemotherapy have already been cured with local therapy alone, there is strong impetus to avoid the possibility of drug-related fatalities. Second, patients in adjuvant trials are by definition asymptomatic from their underlying disease, and therefore many physicians feel particularly keen motivation to minimize the adverse impact of treatment on quality of life.

Along these lines, the evolution of treatment in childhood cancers provides useful counterpoint. Pediatric oncologists have been able to assume that the treatment of a cancer is a child's most important job following diagnosis. After all, children have no dependents and everything else—schooling, recreation—can wait. Although substantial progress in sensitive tumors such as rhabdomyosarcoma (6) and Wilms' tumor (5) was possible with relatively nontoxic therapies, high-dose aggressive treatment was developed and implemented when lesser measures were insufficient and when the miserable prognosis of the underlying disease justified doing so (7,37). The doses (7) and toxic effects (37,38) of some of these regimens are formidable by the standards commonly used by adult oncologists for adjuvant chemotherapy. Although a child or adolescent can probably tolerate some therapies better than adults, particularly elderly patients with significant medical problems in addition to cancer, one still must wonder to what extent the advances in the treatment of childhood tumors are due to pediatricians' willingness to "think cure" and operate at the upper end of tolerable dose when necessary, instead of compromising dose consistently. Is it likely that aggressive regimens would have been developed against osteosarcoma and Ewing's sarcoma if these diseases had their age incidence peak in the fifth or sixth decade?

What is the hard evidence that dose or dose intensity is an important determinant of outcome in clinical adjuvant trials? Definitive answers are simply not available. The only prospective randomized trial examining a dose-response relationship in adjuvant breast cancer (39) compared standard doses of cyclophosphamide, methotrexate, and 5-FU (CMF) to low doses of the same regimen; although the trial revealed no differences, the sample sizes were small and the resultant power was consequently low. Retrospective analyses of the relation of actual administered dose to outcome have been used to support (40,41) and refute (42,43) the importance of dose. Such analyses are probably best regarded as noncontributory because of the methodologic problems associated with them (44).

Most trials of adjuvant breast cancer therapy have employed standard regimens for advanced disease. By any reasonable measure, doses have been in a moderate to low range and conservative attenuation schedules require dose reduction for minor evidence of myelosuppression or other side effects. Table 3 shows the regimens, dose modification schedules, and observed degrees of toxicity for the studies of two cooperative groups during the 1970s. Although these regimens seemed adequately vigorous at the time they were written, the dose intensity of the myelosuppressive agents clearly turned out to be submaximal, and the observed degrees of myelosuppression were quite mild.

Because of the very cautious attenuation schedules of the past decade, nearly all adjuvant studies in breast cancer have documented an inability to administer full projected doses of regimens that are not very intensive to begin with. With occasional exceptions (53), the apparent inability to give full planned doses of adjuvant regimens has been as true of single-center studies as of multicenter trials in the cooperative groups (table 3). Although there has been debate as to whether delay of the next cycle with subsequent administration of full dose is preferable to administration of attenuated doses on time (13), the

fact is that either option results in a lower than projected cumulative dose per unit time. Clearly, certain levels of toxicity legitimately mandate dose reduction or delay. Without doubt, however, the rules for attenuation in most trials have been much more cautious than necessary.

Recent data from the Milan group (13) suggest that CMF for 6 months is associated with fewer downward dose adjustments and with delivery of a higher percent projected dose than is CMF for 12 months. This is not surprising, since the longer the duration of therapy, the more opportunities exist for dose modification and the greater the incidence of cumulative toxic effects that necessitate a dose reduction. In addition, participating physicians may have less tendency to attenuate dose if the total duration of treatment is relatively short.

In summary, conclusive evidence is lacking to support the importance of high doses in the clinical adjuvant setting. The question has not had an adequate prospective test, although Cancer and Leukemia Group B is currently attempting one (47). Nearly all trials in breast cancer to date have used less than maximally vigorous treatment. The universal presence of a dose-effect relationship in laboratory models and the dependence of clinical response rate on dose provide ample rationale for implementation of high-dose regimens in the adjuvant setting.

Table 3: Planned Doses, Dose Modification Schedules, and Selected Toxicity Frequencies of Two Multicenter Breast Adjuvant Studies Utilizing Combination Chemotherapy (all doses are mg/m$^2$)

| | CALGB 7581 (45) | SWOG 7436 (42) |
|---|---|---|
| **Regimens** | | |
| | C 80 po q d<br>M 40<br>F 500 } IV weekly x 5<br>V 1.0<br>P 40 | C 60 po q d<br>M 15<br>F 300 } IV weekly for 1 year<br>V 0.625<br>P 30 po q d 1-4, then taper |
| **Planned Dose Modifications** | | |
| Leukopenia | 50% for WBC 2500-3900<br>Omit for WBC <2500 | 50% for WBC 2000-3999<br>Omit for WBC <2000 |
| Thrombocytopenia | 50% for plts 75,000-100,000<br>Omit for plts <75,000 | 50% for plts 50,000-99,999<br>Omit for plts <50,000 |

**Percent of Patients with Indicated Toxicities**

| | Dose | CMF | CMFVP | Dose | CMFVP |
|---|---|---|---|---|---|
| Leukopenia | Induction 2000-4000 | 80% | 54% | 2500-3999 | - 39% |
| | Induction <2000 | 7% | 13% | <2500 | - 10% |
| | Maintenance <2000 | 7% | 8% | | |
| Thrombocytopenia | Induction <100,000 | 3% | | 50,000-99,999 | - 6% |
| | Maintenance | 15% | 7% | <50,000 | - 1% |
| Mucositis | Induction | 9% | 25% | | - 16% |
| | Maintenance | 11% | 20% | | |
| GI | Induction | 40% | | | |
| | Maintenance | 10% | 16% | | |
| Hematuria | Induction } | ≤12% | | | |
| | Maintenance } | | | | |

| Mean Percent Full Dose Administered | | | |
|---|---|---|---|
| Induction | C | 74.9% | 78.6% |
| | FU | 76.2% | 77.2% |

| Percent Full Dose Administered | |
|---|---|
| C | 70% |
| M | 71% |
| FU | 73% |
| V | 78% |
| P | 84% |

| Percent of Patients Receiving Full Induction Doses of: | |
|---|---|
| C | ~35% |
| FU | ~30% |

Dropout Rate - Not stated

Dropout Rate - 5.3%

Several trials recently activated by the cooperative groups are testing significantly more aggressive regimens (46-48); the doses in these studies are for the moment probably as high as is reasonable to expect in a multicenter setting.

### Selection of a Regimen

The guiding principle over the past 25 years has been that only regimens showing "significant activity" in advanced disease merit testing as adjuvants. This has seemed as reasonable a criterion as any, given that indiscriminate testing of all drugs or combinations is impossible and that activity in advanced disease ought to carry with it some predictive value for activity against micrometastases of the same histologic type and primary site.

What guidance can laboratory models give us on this question? Table 4 shows data on the effectiveness of chemotherapy both in advanced disease and in the adjuvant setting against several murine tumors most commonly used as adjuvant models. In most cases where adjuvant therapy shows striking effects, the same regimen is highly active against advanced disease; response rates in Lewis lung, colon 26, and C3H mammary line 16 are 60%-80%, with 24%-80% of the responses complete. This apparent correlation, however, is not invariable. Adjuvant treatment of B16 melanoma with semustine, which has only an 8% response rate against advanced disease, increases the cure rate from 20% with surgery alone to 70% with combined modality therapy. In interpreting this apparently anomalous result, one must realize that although semustine gives a very low response rate, it results, nevertheless, in a significant increase in lifespan of animals bearing advanced disease.

Additional data suggest that regimens which are without significant activity (in terms of prolongation of life) against advanced disease may be strikingly effective following initial cytoreduction with chemotherapy. In experiments with plasmacytoma Pla 1, Schabel (24,25), has shown that cytarabine, which has little effect when used alone in advanced disease, is curative following a dose of cyclophosphamide. Similar results are obtained after loading dose schedules with several regimens in L1210 leukemia (110). These experiments are not easy to interpret mechanistically, since drug interactions at the cellular level can certainly not be excluded, but they again suggest that a drug's efficacy against advanced disease may be an inadequate predictor of efficacy against a reduced tumor burden.

On the whole, the experimental data suggest that regimens which are active adjuvants are highly active in advanced disease, far more active, in fact, than most clinical regimens are in advanced human cancer. The volume of experimental data available for correlations of this kind is admittedly meager, however, and we particularly lack information on the activity in advanced disease of relatively ineffective adjuvants.

What about the clinical experience? Although response rate has almost always been the parameter used to determine whether a regimen should be tested as an adjuvant,

Table 4: Chemotherapy of Transplantable Murine Tumors: Relation of Results in Advanced Disease to Effectiveness After Surgical Cytoreduction (data from References 15, 24, 25)

| | | | Chemotherapy Alone | | | | Surgery alone | | Surgery followed by chemotherapy | |
|---|---|---|---|---|---|---|---|---|---|---|
| Tumor | Stage | Regimen | % RR | % CR | % Cures | % ILS | % Cures | % ILS | % Cures | % ILS |
| Lewis Lung | 0.4 gm. | CPA | 60 | 24 | 22 | 60 | - | - | - | - |
| | 1.0 gm. | CPA | - | - | 0 | 0 | 0 | 0 | 50 | ++ |
| B16 Melanoma | 0.5 gm. | MeCCNU | 8 | 0 | 0 | 45 | - | - | - | - |
| | 0.5-1.0 gm. | MeCCNU | - | - | - | 64 | 20 | ~100 | 70 | ++ |
| | 0.5 gm. | CPA + MeCCNU | 15 | 10 | 0 | 20 | - | - | - | - |
| | 0.5-1.0 gm | CPA + MeCCNU | - | - | - | - | 0 | - | 30 | - |
| Colon 26 | 0.1-0.6 gm. | MeCCNU | 75 | 40 | 5 | ~114 | - | - | - | - |
| | 0.8 gm. | MeCCNU | - | - | - | ~ 68 | 35 | 155 | 65 | - |
| C3H Mammary Line 16 | 0.1-0.6 gm. | Doxorubicin | 69 | 57 | 0 | ~ 35 | - | - | - | - |
| | 1.0 gm. | Doxorubicin | - | - | - | - | <20 | - | 15-50 | - |
| | 0.1-0.6 gm. | Melphalan | 80 | 80 | 0 | 43 | - | - | - | - |
| | 1.0 gm. | Melphalan | - | - | - | - | <20 | - | 40 | - |
| Spontaneous Mammary Adenocarcinoma in $CD_8F_1$ | | STEAM | - | - | - | - | <5 | - | 30 | - |
| | | Uracil Mustard | - | - | - | - | 9 | - | 27 | - |

Table 5: Relation Between Response Rate in Advanced Disease and Efficacy as Postoperative Adjuvant

| Disease | Regimen | Response Rate | Results of Adjuvant Trials (Ref) |
|---|---|---|---|
| Breast | L-PAM | 20-25% | Positive (12) |
| | CMFVP | 40-60% | Positive (42) |
| | CMF | | Positive (13) |
| Melanoma | DTIC | 9-30% | Negative (51) |
| Colorectal | FU | 12-20% | Negative (49) |
| | Methyl CCNU | " | Negative (50) |
| | FU | | |

there has been no uniformly accepted threshold response rate above which a regimen has been consistently judged to merit adjuvant testing. The decisions have related much more to contemporary notions of what constitutes state-of-the-art therapy for the particular disease. Table 5 shows approximate response rates of selected regimens in advanced disease and efficacy of those regimens in the adjuvant settings, where the regimen has been used postoperatively for at least several cycles and compared against either a positive or a no-treatment control. For regimens of low activity, response rate in advanced disease is not a good predictor of adjuvant efficacy; although the activities of 5-FU in colon cancer, dacarbazine in melanoma, and melphalan in breast cancer are in the same range, the first two have produced no statistically significant benefit as adjuvants in individual trials (49-51). On the other hand, the totality of available data, including the pediatric experience, certainly does suggest that markedly effective adjuvants have high response rates in advanced disease. When response rates become very high, however, as in testicular cancer and Hodgkin's disease, the very effectiveness of the regimen as salvage therapy may obscure any benefit of the same regimen used as an adjuvant.

In the absence of specific hypotheses justifying otherwise, therefore, we should probably reserve for adjuvant testing those regimens that have demonstrated activity in advanced disease. If anything, the preclinical and clinical adjuvant data suggest that we need to develop more stringent criteria than a reasonable response rate for deciding whether a regimen merits testing as an adjuvant. A high response rate merely indicates that a regimen exerts cytotoxicity against a high proportion of tumors. Without additional information such as a high complete response rate, long duration of response, or prolongation of survival, a high response rate itself does not indicate that the degree of tumor cell kill has been sufficient to produce a clinically useful effect.

The question of regimen selection has additional complexities, however, and we shall return to it in the discussion of suppression of resistance.

### Timing of Treatment

Clinical surgical adjuvant trials generally allow a certain period of time to elapse between surgery and the start of chemotherapy. In most breast cancer studies, for example, patients may still be accrued if the time from surgery to commencement of chemotherapy does not exceed 4-10 weeks. The obvious assumption underlying such a flexible policy toward the start of systemic therapy is that delay is not critical to outcome. After all, why should it be? The age of a human tumor from malignant transformation of the first cell to the time of clinical detection is surely measured in months or even years. What possible difference to outcome can a few weeks' delay make?

In laboratory models, generally the earlier treatment is begun in relation to surgery, the better the outcome (24,25,54,55), although not always (27). In most systems, delays of a few days result in much lower cure rates than the same treatment begun within 24 hours of resection of the gross disease. Because of the relatively rapid tumor volume doubling times (1-2 days) of murine tumor models in early stages (14), these results have until recently been regarded as simply reflecting an increasing tumor burden with each day's delay in therapy.

Other mechanisms may be at work here as well. Several groups have shown that in a wide variety of model systems an interplay exists between the growth of individual tumor implants and the total body tumor burden. It has been known for about 15 years that a decrease in body burden of tumor decreases the tumor mass doubling time in sarcoma 180 and adenocarcinoma 755 (56). Reduction of body burden can shorten the cell cycle time in human acute lymphoblastic leukemia (ALL) (57) and

ovarian cancer (58). More recently, Simpson-Herren et al (59) have shown that resection of gross Lewis lung carcinoma can result in an increase in growth of residual metastases and a decreased median survival time; the increased growth is associated with an increase in labeling index (LI) and tumor growth rate rather than with a change in cell-cycle or S-phase times. Artificial metastases produced by iv injection of tumor cells have their growth rate slowed by sc implantation of a second tumor; the survival of such doubly implanted mice is greater than that of mice given iv tumor cells only.

Gunduz et al (60) have found similar behavior in the C3H mammary tumor system. Following implantation of two tumors and then resection of one several days later, the LI in the residual focus increases for about 10 days, with an increased tumor doubling time, but with minimal changes in DNA synthesis time and cell cycle time. A measurable increase in tumor size is apparent a week after resection of the first tumor.

In studies of two implants of unequal size (61), removal of the larger tumor (the "primary") increases the LI of the smaller one (the "metastasis") by about 55% of control by Day 3; this effect is dissipated by Day 7. The increase in LI is accompanied by an acceleration in growth of the residual tumor. This increase in LI induced by tumor resection can be abrogated by cyclophosphamide given on the day of surgery (Day 0) in a dose-dependent fashion; doses of 60 mg/kg give an initial increase in the LI on Day 1 (an increase greater than that expected from surgery alone), followed by subsequent depression of LI. If > 60 mg/kg is administered, the LI is uniformly depressed at all subsequent time points; tumor growth curves parallel the kinetic changes. If cyclophosphamide is given on Day 3 rather than Day 0, one sees similar curves for all doses but longer and deeper depressions of LI at the higher doses. Maximum growth retardation is achieved by giving the drug 5 or 7 days before surgery. Survival is also best when cyclophosphamide is given at or before surgery; survival is prolonged during the first 4 weeks with a subsequent increase in the death rate after that time.

Whether resection of bulk tumor in humans can cause acceleration of residual disease has been discussed in the literature from time to time (62,63). Any seasoned oncologist can draw from his own storehouse of anecdotes at least a case or two in which a surgical procedure seems to have been followed by an increased pace of tumor growth compared with the preoperative rate. Needless to say, such cases may be more memorable than those in which growth of disease has accelerated without obvious predisposing cause. Laboratory experiments to examine the effect of surgery on metastatic tumor growth have indeed suggested that metastases are larger after resection of the "primary" than in the absence of surgery (64-68). Several of these experiments, however, did not correct for the fact that animals may live longer after surgery than without it (65). The effect of surgery on number of metastases has been inconstant; studies have documented both an increase (64-66) and decrease (67,68).

For our purposes here, the issue is the complex potential interaction between surgical resection and the timing of chemotherapy. If the phenomena described by Fisher and Simpson-Herren have a clinical counterpart, it is important to recognize that such effects (increase in LI and a parallel acceleration in tumor growth) would be ordinarily impossible to recognize with casual observation alone. Fisher has clearly shown, however, that although these effects are quite transient in the models, they have important therapeutic implications concerning the timing and dose of chemotherapy.

In addition to an effect of surgery on growth of residual disease, the possibility that the trauma of a surgical procedure might enhance tumor cell dissemination has worried clinicians for some time. In fact, the first multicenter breast adjuvant trial was based upon the hypothesis that chemotherapy might sterilize cells released into the general circulation at surgery. Tumor cell invasion into the vasculature has been appreciated for decades (69). Release of cancer cells into the circulation can be increased by palpation, manipulation of the tumor during surgery or radiotherapy, or surgery itself (70-73). Although the physiological and medical significance of these observations has been questioned (74,75), there is some suggestion that surgical techniques which isolate the tumor's vascular supply result in a better outcome (76). Where possible, current standard surgical practice appears to emphasize minimal manipulation of the tumor, as well as isolation and ligation of the tumor's blood supply before resection (77). If, in fact, enhanced tumor cell release during surgery has clinical significance, the possibility that perioperative therapy might interfere with the process of metastasis itself seems as reasonable today as it did in the 1950s.

What of the clinical experience? Despite physicians' general disregard for the importance of timing, this parameter is considered quite seriously in some clinical contexts. Pediatricians are accustomed to regarding the diagnosis of Wilms' tumor almost as a medical emergency. Retrospectively, analyzed data from the early experience with dactinomycin therapy suggested that delays of even a few days might be highly prejudicial to outcome (78). Since nephroblastoma usually grows very rapidly, the dependence of outcome on early institution of treatment was regarded as plausible, and starting therapy within hours of surgery has become standard procedure.

Two well-known trials in breast cancer have also employed immediate postoperative treatment. In both cases, the hypothesis was that such treatment would neutralize surgically induced dissemination of tumor cells into the systemic circulation. Both trials employed single alkylating agents. The Scandinavian multicenter study (79,80) used cyclophosphamide in a single 5-day course. The vast majority of patients randomized to cyclophosphamide treatment had therapy instituted within 24 hours of sur-

gery. Despite the relative ineffectiveness of cyclophosphamide as a single agent against advanced breast cancer, and despite the fact that only one course of treatment was given, the treated population had a statistically significant improvement in survival compared with the no-treatment control. In the one participating institution in which chemotherapy was given after a several-week delay to permit delivery of radiotherapy, the chemotherapy-treated group did no better than control. In all other participating institutions, which employed immediate treatment, outcome was better with chemotherapy than without. Along similar lines, the very first multicancer breast adjuvant trial in the United States (1,81) employed thiotepa in a perioperative fashion; results suggested probable benefit for the subgroup of premenopausal patients with more than three positive nodes (81,82).

Because the differences in survival between treated and control groups were of modest magnitude (79,82) or confined to a subset of patients (81), these trials were generally regarded as disappointing, and the oncology community has been reluctant to follow up on what should have been regarded as a promising lead. Currently, the Ludwig Clinical Trials Group is conducting a prospective randomized study of immediate postoperative treatment. Attempts to define the importance of timing retrospectively have led to analyses in which results are examined as a function of interval between surgery and start of chemotherapy (42,83). Such analyses are problematic, since they cannot control for potentially important selection biases that may have determined why some patients were started early and others were not. In addition, since the vast majority of patients in completed breast cancer trials to date were started on therapy at least 2 weeks postoperatively, these analyses are really comparing various degrees of lateness, rather than early versus late start of treatment.

By no means have all perioperative trials been positive. The peri- and postoperative use of alkylating agents singly in patients with non-small cell lung cancer is no better than observation (84–86). One course of floxuridine as an immediate adjuvant to surgery in resectable colon cancer is ineffective (87,88), as is intraluminal administration of 5-FU at the time of operation (89). Interpretation of the strikingly positive study of Taylor et al (90) of intraportal administration of 5-FU beginning at the time of operation is clouded by the uniqueness of the route; attempts to confirm these results are in progress within the National Surgical Adjuvant Breast Project (NSABP). Although it is tempting to dismiss the negative trials because of the general ineffectiveness of the chemotherapy used in them, the results serve to emphasize that the use of perioperative drug administration will not by itself convert ineffective single agents into effective adjuvants.

Let us now turn to the obstacles impeding large-scale clinical testing of early institution of therapy.

*Preoperative chemotherapy.*—Here there are really no obstacles; in fact, the past few years have seen a very great upsurge of interest in the initial use of chemotherapy as part of complex multimodality treatment models. This has stemmed from the hope that improved local tumor control can be achieved by the same maneuvers that will also control distant metastases. Most such studies, therefore, have concentrated on tumor types for which significant local control remains a problem or for which certain dilemmas in local treatment (eg, limb salvage) are very pressing. Sarcomas of bone, carcinomas of head and neck, and more recently, locally advanced breast cancer have been the focus of innumerable pilot studies and even a few large-scale trials. The promises and problems of this general approach have been discussed in detail elsewhere (91) and have been the subject of much recent attention at international meetings.

*Immediate postoperative chemotherapy.*—Several types of practical problems have conspired to prevent wider testing of regimens begun immediately after surgery.

(*a*) With immediate therapy the physician is necessarily unaware of the complete pathologic stage of the patient. The approach is, therefore, impossible if the suitability of a patient for treatment depends upon this information. With breast cancer at least, this issue is probably less pressing than it used to be, as trials in the negative node subset are now widely regarded as justifiable.

(*b*) Immediate therapy requires a degree of administrative coordination that challenges the flexibility of many hospitals and physicians. Insofar as staging for distant metastases is important, these studies must be done preoperatively in an expeditious manner. Surgeon and medical oncologist must mesh schedules so that each knows what the other is doing and when.

(*c*) Many of the well-known acute toxic effects of chemotherapy would be most unwelcome intruders in the postoperative period. Nausea and vomiting associated with many drugs would probably be intolerable for a patient with a fresh abdominal or thoracic incision. Heavy doses of antiemetics or sedation with chemotherapy might alleviate these side effects, but only at the risk of increased postoperative pulmonary complications. The induction of significant myelosuppression within 7–14 days of major surgery would also carry significant risk, particularly after resections in potentially contaminated operative fields. For these and other reasons, there are significant constraints on the kinds of regimens that can be employed safely in the immediate postoperative period. The constraints are doubtless stricter for visceral than for superficial operations and would obviously also depend on the age and incidence of major co-morbidity in the study population.

(*d*) Immediate chemotherapy might prevent adequate wound healing. The specter of late dehiscence or of a cosmetically unacceptable result haunts consideration of this approach. A fair body of experimental evidence in-

dicates that many classes of chemotherapeutic agents can significantly reduce the strength of surgical wounds (92-96). Alkylating agents, antimetabolites, anthracyclines, anthraquinones, and glucocorticoids have all exhibited an effect in various experimental models. Generally, the effect is greatest when the drugs are administered in the immediate perioperative period (92,93); drugs administered after substantial wound healing has already occurred have little or no effect. Certain studies with doxorubicin and mitoxantrone, however, have indicated a greater effect when the drugs are administered 7 days preoperatively than on the day of wounding (96).

Whether effects on wound healing will be of clinical importance probably depends on the choice of regimen and its scheduling in relation to surgery. Thus far, an extensive experience with various preoperative chemotherapy regimens in several tumor types has revealed no obvious problems with wound healing. The experience with immediate postoperative treatment is far more limited. Reports from the completed perioperative trials in breast cancer reveal no increase in complication rate (79-81). An early report of the toxicity associated with perioperative CMF by the Ludwig Group does, however, suggest an increased incidence of several important side effects of chemotherapy in the group receiving immediate postoperative therapy (97); these have included wound healing disorders and local infection. The increased incidence of infection has led to some modifications in patient eligibility requirements and in the techniques of methotrexate administration; no subsequent results from this trial have yet been published. As with other side effects of chemotherapy, problems with wound healing are likely to have greater clinical consequences after major thoracic, abdominal, or head and neck procedures than after relatively superficial operations like breast cancer or melanoma surgery.

(*e*) In cases where a firm diagnosis cannot be made preoperatively, immediate chemotherapy may be impossible if the patient insists on an interval between diagnosis and definitive therapy for discussion of therapeutic options and possibly second opinions. With the widespread use of needle biopsy for diagnosis, this is probably not an important consideration at most primary sites.

### Body Burden

The inverse relation between body burden of tumor and probability of cure with adjuvant therapy is a constant finding with transplantable tumors. The longer the interval between implantation and start of therapy or between surgery and chemotherapy, the lower the cure rate (14-16,21,24,55,98,99). Whatever the biological basis for this effect, it is a quantitatively important one in murine models. In the clinic, of course, body burden at diagnosis is not a controllable variable, except to the extent that early detection programs increase the prevalence of relatively early disease and that therapy is instituted as expeditiously as possible after diagnosis.

Although there are no precise techniques for estimating body burden of most tumors, relative body burden within a given tumor type is probably roughly mirrored by locoregional stage. The clear separation in time-to-recurrence curves for the various TNM stage groups in most tumors indicates that occult disease associated with low-stage tumors takes longer to become clinically manifest than high-stage disease. Since in addition the different TNM stage groups generally have clear differences in cure rates, it seems most likely that body burden at diagnosis is less with low-stage than with high-stage disease.

Is there good clinical evidence that human tumors of relatively low stage are more susceptible to the effects of adjuvant therapy than higher but still operable stages? The data on this point are confusing, and the use of interim trials data to answer this question may compound the confusion, since early-stage disease has a later relapse-time distribution than more advanced disease. The experience of both the NSABP (12) and the Istituto Nazionale Tumori (13) suggests that chemotherapy is relatively more effective in patients with N1-3 than in those bearing four or more positive nodes. The relative superiority of CMFVP (cyclophosphamide, methotrexate, 5-FU, vincristine, and prednisone) over melphalan in Southwest Oncology Group 7436 (42) appears greater for N1-3 (10% vs 29% recurrences) than for $N > 3$ (27% vs 46% recurrences). Certain preliminary results in very favorable (T1N0) node-negative subsets in breast cancer look extremely good (83), although admittedly these data are early and difficult to interpret without control groups.

On the other hand, the effect of chemotherapy in the Nissen-Meyer trial appears at least as great in the N(+) as in the N(-) group (79,80). In NSABP B07 (12), PF appears superior to P alone in patients with age 50 and $N > 4$. Promising early results for the node-negative groups in the Swiss OSAKO trial have disappeared with continued follow-up (100); the N(+) patients, however, continue to show a superior relapse-free survival bordering on statistical significance. Most data from gastrointestinal cancer adjuvant trials suggest no convincing dependence of efficacy on tumor stage. While some interim analyses have suggested greater effect in less advanced subsets, the Gastrointestinal Tumor Study Group's positive study of semustine and 5-FU in gastric cancer shows, if anything, greater significance for survival differences in subgroups with positive nodes and with adjacent tissue invasion than in the correspondingly more favorable subgroups (101). The Stanford experience in the multimodal therapy of Hodgkin's disease suggests superiority for combined modality therapy [radiation followed by MOPP (mechlorethamine, vincristine, procarbazine, and prednisone)] only in a relatively poor-risk subgroup; pathologic stages I and II do not appear to benefit significantly (101). The Hodgkin's disease experience is of uncertain relevance in the present context, since the extent of local

therapy itself varies with stage and takes many weeks to execute.

Since virtually none of these trials was designed with sufficient power to detect modest subgroup differences, any conclusions from this kind of exercise are provisional at best. At this point in time, it would appear that within the subset of patients who are candidates for inclusion in adjuvant protocols (ie, those whose locoregional disease can be resected with no grossly detectable residual), it is not clear that tumor burden, as reflected by locoregional stage, is a major determinant of treatment efficacy. The lack of clear evidence on this point, however, may be due to the absence of adequate statistical power mentioned above.

### Suppression of Resistance

The greater efficacy of combinations of drugs compared with single agents has been apparent for years from laboratory and clinical studies. Progress in developing curative therapies for several sensitive tumor types has shown that the complete remission, widely regarded as a prerequisite to cure, is much more likely with combinations than with single drugs.

During the 1970s, attempts to increase the efficacy of combination chemotherapy led to the use of combinations in sequence. The motivation for these designs was largely empirical and qualitative but nevertheless seemed quite compelling: if two drug combinations used independently each had a certain level of effectiveness, and if the two combinations were at least partially non-cross-resistant, then the use of both combinations together in alternating sequence from the beginning of therapy (ABABAB . . .) ought to be more effective than the use of either one to failure. In addition, some such trials employed rapid alternation in sequencing (102); it was reasoned that since responses are maximal within a few weeks of start of therapy, an optimal schedule should expose the tumor to as many active agents as possible within this crucial period.

Trials of alternating combinations now fill the literature. Results from the vast majority in most tumor types have not suggested significant improvement over what is attainable with standard combinations. In a few notable circumstances, however, the use of this strategy appears to offer an advantage over conventional administration (103–105). From the experience with this approach, three simple observations have been clear for some time:

(*a*) Most trials of this kind were begun without information concerning the extent to which the alternated combinations were in fact non-cross-resistant. In the construction of alternating regimens, the fact that different drug classes were used in the alternated combinations was by itself often taken as prima facie evidence of lack of cross-resistance. As has become very plain within the past few years, however, patterns of drug resistance are often impossible to predict from structural or mechanistic considerations alone (106).

(*b*) Apparent success has been clearly shown so far in Hodgkin's disease (103), and also seems likely in the aggressive non-Hodgkin's lymphomas (107), both tumors in which appreciable complete remission rates are possible with standard combinations. Tumors for which existing combinations are capable of inducing at most partial responses have not yet shown clearly improved results with an alternating strategy.

(*c*) In many instances, the nonrandomized nature of many of these trials, and the rather low power of many of the randomized ones, make clear conclusions impossible purely on methodological grounds.

As these clinical efforts were proceeding, developments in the laboratory and in theoretical modeling have done much to clarify possible mechanisms and suggest techniques for suppressing the emergence of drug resistance more effectively. Goldie and Coldman (108,109) have developed a mathematical model of the development of resistance to chemotherapy. Based on certain simple assumptions about the genetic origins of drug resistance, the model's principal predictions agree with much experimental information from murine tumors. Several of these predictions are also either widely acknowledged clinical facts or else strongly suggested by at least some clinical trials (112): (*a*) small tumor burdens are more curable than large; (*b*) tumors which are identical histologically may have very variable responses to identical regimens at equivalent tumor burdens; (*c*) curability may be lost over a small (about 2 log) increment in tumor burden; (*d*) combination chemotherapy is superior to single-agent treatment; (*e*) the early utilization of all known effective agents is superior to a strategy in which some active agents are saved for later use as maintenance; and (*f*) alternating non-cross-resistant regimens should be superior to the use of one regimen until failure and then the other, provided that the combinations being alternated are of equivalent efficacy. This latter finding may point to a crucial additional failing of many of the clinical trials; evidence attesting to the therapeutic equivalence of the combinations used in alternating sequence has almost always been lacking. In fact, the most striking example of the success of the alternating approach happens to be the one for which therapeutic equivalence was most convincingly demonstrated prior to the alternating trial (103). Elsewhere in this issue, Goldie and Coldman discuss in detail some of the implications of the somatic mutation theory for the construction of clinical combinations.

The more recent refinements of the somatic mutation theory (110,111) have very interesting implications for our purposes here. Many of the relatively resistant human tumors are characterized by a low growth fraction with an appreciable amount of cell loss during growth. The number of generations from the initial transformational event to the appearance of clinically apparent disease is vastly greater if cell loss is a major feature of

tumor growth than if it is not. Since for a given mutation rate the number of random mutational events accumulated by the surviving cell population will depend on the number of elapsed generations, it is clear that the greater the genetic age of a tumor, the more likely the tumor is to have accumulated mutations to drug resistance.

This may mean that, given our present collection of drugs, the accumulation of multiply resistant phenotypes is so great by the time a tumor has manifested gross metastases that no combination of available agents is likely to increase complete response rates significantly in advanced disease. Whether one accepts this depressing scenario or not, however, the Goldie-Coldman model gives additional force to the potential importance of treating presumed micrometastases as soon as possible after surgery. A delay of several weeks is well within the range that the somatic mutation theory predicts may permit the evolution of drug-resistant variants from drug-sensitive parental lines.

The model suggests, then, the hypothesis that the almost universal drug resistance of many common cancers may be a characteristic acquired during the course of tumor growth and may not be a feature of the early evolution of the tumor. If this is so, it is at least possible that a strategy which uses drug combinations as early as possible may permit the extinction of the still sensitive malignant population. The viability of this hypothesis depends on when in the course of tumor growth such mutations accumulate relative to the time of diagnosis. It goes without saying that such drug combinations will have to be selected by criteria other than conventional levels of activity in advanced disease. We shall return to this point later.

It must also be emphasized that the application of the somatic mutation theory to the clinic is not restricted to the use of alternating combinations, at least not in a rigidly interpreted fashion. Goldie and Coldman have stressed that the essential features of the model require only that the maximum number of active agents be used as early in therapy as possible. This requirement would be best satisfied, of course, if all active agents could be given together and repeated just as soon as toxicity permitted. Strategies like alternating combinations become necessary only because drugs with overlapping toxic effects cannot generally be given over a very tightly compressed time period. Illustrations of other ways of translating the implications of the somatic mutation theory to the clinic are provided by recent protocols from the Vancouver group, the results of which are quite encouraging (113).

## Suggestions for Future Trials

The development of effective clinical adjuvant treatments with cytotoxic agents has reached a critical point. The last two decades have taught us two invaluable lessons: (*a*) successful clinical adjuvant regimens are indeed possible; and (*b*) striking improvements in the treatment of the common epithelial tumors of adulthood will not come easily. They are most unlikely to result from minor variations of previously tested regimens.

The oncology community has two major options at this point. The first is to decide that existing tools have an insufficient therapeutic index to do the job. Because the drugs are simply not as active against human tumors as against murine ones, and because a whole series of practical constraints inhibits the clinical oncologist from doing with his patients what the experimental chemotherapist routinely does in his laboratory, the models do not apply to the vast majority of human tumors. The logical conclusion of this argument is to cease attempts at developing effective surgical adjuvants until a much more effective repertory of drugs becomes available.

The second possibility is to try much harder than we have thus far to implement the principles learned in experimental and theoretical work, as outlined above. To do this, demonstration of the feasibility of new approaches will be necessary. Primarily because of the need for large sample sizes, cooperative groups are probably the only realistic mechanisms for performing definitive adjuvant trials. Before a new generation of such studies is launched, however, we need a series of small pilot studies in single centers whose goal is to develop feasible and tolerable regimens designed to test the hypotheses outlined previously. I believe that the following suggestions are amply justified by the laboratory and clinical experience over the past decade.

(*a*) We must change our standards of "tolerability" for adjuvant regimens. Oncologists are often unjustly criticized for ignoring the quality of life of their patients. On the contrary, a veritable preoccupation with side effects of treatment and quality of life has probably inhibited the clinical testing of many worthy approaches. An emphasis on quality of life is completely appropriate once really effective therapies are at hand; the current effort of the National Wilms' Tumor Study Group is an excellent example of how quality of life questions should be integrated into research programs. A risk-benefit estimation, once the necessary information is available to make one, is certainly an appropriate determinant of whether a therapy should be placed into widespread use outside the context of a clinical trial. But to permit quality of life issues or fear of early or late treatment-related toxicity to paralyze the generation of innovative clinical trials aimed at developing new strategies is totally counterproductive. In this regard, a meaningful informed consent process permits the patient to be a partner in the decision and relieves the physician of the immense burden that would result from making decisions alone. For his part, of course, the physician can in good conscience participate in trials only if he feels that the experiment is scientifically meritorious and stands a good chance of conferring benefit on the patient.

(*b*) Dose intensity should be increased significantly be-

yond what is conventionally considered appropriate for adjuvant studies. Aggressive adjuvant regimens can be piloted in poor-risk patients (eg, breast cancer patients with multiple positive axillary nodes) and regimens which prove tolerable may be moved up to a better prognostic group. The initial studies of this kind should be phase I in design and intent; that is, the individual drugs in a combination should be escalated stepwise to yield a true MTD for the combination. Groups having different medical risks for such treatment should probably be approached separately (eg, the age or menopausal groups in breast cancer). If physicians have qualms about performing dose-finding studies even in a poor-risk adjuvant setting, they may be performed instead in patients with advanced disease. The disadvantage to doing so is that the MTD in the minimum residual disease setting may be different from that achieved in the presence of gross disease.

Myelosuppression should not be regarded as dose-limiting unless absolute granulocyte counts fall $< 1000/mm^3$ or platelet counts fall $< 30{,}000/mm^3$. With the induction of chloruresis, doses of cisplatin can be increased beyond 120 $mg/m^2$. Agents whose limiting toxic effects include mucositis can be pushed until this effect is seen in a much higher proportion of cases than has been true in most studies to date. Doxorubicin administration can probably also be intensified by increasing dose rate per unit of time. Although the relationship of scheduling to incidence of cardiotoxicity has not been completely clarified (114), it seems reasonable in this setting to adopt continuous infusion or weekly schedules to minimize the probability of cardiomyopathy with escalating doses.

Serious consideration should be given to piloting the use of high-dose chemotherapy with autologous bone marrow reconstitution in certain refractory tumor types where data in advanced disease suggest activity. Studies suggest that melphalan and carmustine have greater antitumor activity in melanoma (34,35) and colorectal cancer (33) at high dose than at conventional doses. Responses are almost always partial, however, and response durations are distressingly short. It seems most unlikely, therefore, that the natural history of these diseases will be altered by the high-dose treatments attempted thus far. It *may* be that the use of this modality in the adjuvant setting may be more promising, although the clinical and laboratory studies referred to earlier admittedly make this uncertain. On the other hand, studies at the Dana-Farber Cancer Institute have shown very interesting degrees of activity for combinations of alkylating agents at high dose; patients with a variety of solid tumors have shown a high response rate, including complete responses, many of which are quite durable (115). Such a regimen will be suitable for adjuvant testing, once the toxicity problems have been ironed out. Even if therapy is associated with marked acute side effects and hospitalization, the burdens of treatment, both medical and financial, might well be lower than those of the customary long outpatient programs now in use.

(*c*) Immediate postoperative therapy should be the subject of pilot studies in several tumor types. Breast cancer has already shown itself susceptible to such an approach and is the most obvious tumor candidate for increased attention. Ovarian cancer is also a possibility, particularly with intraperitoneal regimens, the doses of which can easily be adjusted to minimize systemic toxic effects in the immediate postoperative period. Since the peritoneal cavity is overwhelmingly the region at highest risk of recurrence, less than maximal intraperitoneal doses can still preserve a large pharmacological advantage over systemic treatment against peritoneal implants without prohibitive systemic side effects.

Melanoma is also a possible target for immediate therapy. To test the hypothesis outlined earlier, one should not only utilize immediate postoperative (or even preoperative) therapy, but also should include patients with relatively low-stage disease at the time of diagnosis (for example, one to three positive nodes). Parenthetically, it would be of great interest to attempt a direct test of the underlying hypothesis here by comparing drug sensitivities of melanoma cells from very early disease, tested directly by in vitro techniques, to that of cells from late-stage patients.

Selection of a suitable regimen for such trials is a real problem, since the nature of the hypothesis itself precludes the usual clinical guidelines. Perhaps the strongest candidate regimens are the high-dose alkylating agents referred to earlier (34,35,115). The fact that responses to these treatments in advanced disease are only partial and are of short duration suggests that the prevalence of resistant cells in gross tumor is very high. Since, however, many of these partial responses show very impressive degrees of cytoreduction and since we also know that for certain transplantable tumors the observable extent of tumor shrinkage often underestimates by several logs the amount of stem cell killing by treatment (24,116), there is reason to believe that high-dose treatment used in the early postoperative period in early-stage patients might encounter a much decreased prevalence of resistance phenotypes. Although many may regard the possibility of success here as slight, the proposal is based on a reasonable hypothesis and seems worth a good test.

In clinical circumstances where immediate and aggressive postoperative therapy is probably not feasible (notably with visceral cancers that require major thoracic or abdominal procedures for resection), vigorous preoperative treatment with continued therapy postoperatively as soon as possible may be the most promising approach. This strategy is being intensively explored now in osteosarcoma, head and neck cancer, and esophageal cancer. In view of the substantial activity of new drug combinations in bladder cancer (117), this tumor seems a good choice for intensified efforts. Non-small cell lung cancer has already received some attention along these lines; although uncontrolled results to date do not appear particularly

promising (118), patient selection has emphasized those with advanced locoregional disease.

(*d*) For tumors where local control remains a major problem, trials should be directed toward the proper integration of postoperative chemotherapy and radiotherapy. The laboratory studies of Looney, whose studies are summarized elsewhere in this volume, have suggested that optimal results may be achieved by proper sequencing of these two modalities. Although these experiments have been restricted largely to only one model system and have concentrated on cyclophosphamide alone as the chemotherapy of interest, the advantage of sequenced alternated treatment appears substantial. The most obvious candidate for this approach, applied to the adjuvant setting, is resectable head and neck cancer.

(*e*) With regimens of increasing treatment intensity, it will be both necessary and reasonable to shorten the duration of treatment significantly. There is nothing magical about 1-2 years as the optimal length of therapy. When duration of therapy has been examined in oncology, shorter treatments have often proved as efficacious as longer time periods (13,120,121). In terms of patient acceptance, compliance, and objective tolerance, the chance of successfully implementing some of the strategies outlined here will likely depend on the use of relatively short overall treatment durations.

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# Unresolved Issues in Combination Chemotherapy

**Robert E. Wittes* and Abraham Goldin[1,2]**

When the history of medical oncology is written years hence, the three decades from the 1950s to the 1980s will probably be remembered chiefly for the demonstration that systemic treatment with cytotoxic drugs can cure cancer. In view of the difficulties in demonstrating therapeutically exploitable biological differences between tumor cells and normal host cells, this is an almost astonishing finding. It is attributable largely to the discovery and development of a number of active single agents and, no less importantly, to their intelligent use in combination. As is well appreciated, the evolution of curative chemotherapy, used either as a single modality or in association with surgery and/or radiation, has depended heavily on the development of multidrug regimens. In 1985, about a dozen cancers in their disseminated form may be approached with curative intent. In several other cancers, chemotherapy occupies a pivotal role in multimodal treatment strategies for localized disease with a high probability of occult metastases.

We must also admit, however, that chemotherapy has thus far had little or no impact against some of the most common malignancies that afflict mankind. With allowances for vagaries in the natural history of these diseases, the diagnosis of disseminated tumor is still all too often tantamount to a death sentence. Fifteen to 20 years ago, when the promise of combination chemotherapy had yet been largely unexplored, possibilities for progress seemed limited only by the number of studies that could be undertaken. With accumulation of much clinical experience since then, we have been forced to admit that the strategies that have worked against the lymphomas and testicular cancer have yielded little against the carcinomas of large bowel and lung.

Because tangible progress appears to have slowed, many perceive that we are on a plateau of therapeutic achievement and assert that only fundamental advances and the development of much more effective antitumor agents are likely to get us moving again. Although basic biologic insights and new effective agents are indeed necessary, this assertion oversimplifies our present difficulties, which are complex and quite likely to yield at least partially to a variety of solutions. In this paper, we shall examine in a general way where we are in the development of combination therapy with standard cytotoxic agents; we shall not discuss biochemical modulation, which is treated elsewhere in this symposium. We pay particular attention to the interplay between the preclinical laboratory and the clinic. Our perception is that laboratory results have contributed significantly to clinical progress in a variety of ways. We examine the nature of the interaction between experimental chemotherapist and clinician and ask particularly whether some potentially fruitful territory remains to be explored.

## STATE OF THE ART

In table 1, we have divided the commonly treated human cancers into three categories according to the relative effectiveness of present-day systemic therapy against grossly disseminated disease. Included in Group A are diseases for which complete response rates to chemotherapy are very high. Treatment clearly prolongs survival and produces a significant number of cures.

At the other end of the spectrum, Group C includes tumors for which chemotherapy has thus far made no significant and reproducible impact on natural history. Response rates are low (< 40%), complete responses are anecdotal, and prolongation of survival is undocumented. Unhappily, a number of these tumors are very common.

Occupying the very large middle ground are a number of tumor types which have tantalized oncologists for years (Group B). Response rates to best available therapy vary from modest to quite high (40% to ≥ 90%). The proportion of responses which are complete varies from negligible to > 50% in the most responsive tumors of this group. Despite this, however, cure of grossly disseminated tumor is a rare event with chemotherapy alone, even if it does prolong survival in many of these diseases.

Two further observations are pertinent in the present context. The first relates to the possible importance of

[1]Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

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* *Reprint requests to:* Robert E. Wittes, MD, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Landow Bldg, Rm 4A22, National Institutes of Health, Bethesda, MD 20892.

TABLE 1.—Effect of systemic chemotherapy on grossly disseminated cancers

***Group A***—High complete response rates, prolongation of survival, cures
- Acute leukemia
- Hodgkin's disease
- Non-Hodgkin's lymphoma (intermediate and aggressive)
- Germ cell tumors
- Wilms' tumor

***Group B***—Response rates 40%–90%, complete response rates < 50%–60%, cures rare
- Rhabdomyosarcoma
- Non-Hodgkin's lymphoma (indolent)
- Small cell carcinoma of lung (extensive)
- Ewing's sarcoma
- Breast carcinoma
- Head and neck cancers
- Bladder carcinoma
- Soft tissue and bone sarcomas
- Cervix carcinoma
- Prostate carcinoma

↑ roughly increasing complete response rates within Group B

***Group C***—Response rates < 40%, no demonstrable effect on survival
- Gastric carcinoma
- Non-small cell lung carcinoma
- Hypernephroma
- Pancreatic carcinoma
- Hepatocellular carcinoma
- Melanoma
- Colorectal carcinoma

TABLE 2.—Response rates of selected single agents in some "sensitive" tumors*

| Disease | Drug | Response rate |
|---|---|---|
| Hodgkin's disease | Mechlorethamine | 63 |
| | Vincristine | 60 (36) |
| | Vinblastine | 68 (30) |
| | Procarbazine | 69 (38) |
| Testicular cancer | Cisplatin | 60 (19) |
| | Vinblastine | 42 (15) |
| | Bleomycin | 43 (11) |
| Acute leukemia (nonlymphoblastic) | Daunorubicin | 40–50 |
| | Cytarabine | 25 |
| Acute leukemia (lymphoblastic) | Prednisone | 70 |
| | Vincristine | 60 |
| | L-Asparaginase | 60 |
| | Cyclophosphamide | 40 |
| | Daunorubicin | 30 |

* Response rates are in %. For Hodgkin's disease and testicular cancer, the values in parentheses are complete response rates, where available. All data for acute leukemia are complete responses only. *Data from* refs 5–8 and 79.

chemotherapy integrated into combined modality strategies. Adjuvant chemotherapy has not yet demonstrated significant efficacy against tumors in Group C, and the rationale for further trials in the absence of specific hypotheses seems very weak without more effective drugs. Paradoxically, in the solid tumors of Group A a positive effect of adjuvant chemotherapy is also inapparent except in some subgroups; the reason here, of course, is that the striking effectiveness of chemotherapy as salvage following failure of local therapy obscures the impact of adjuvant chemotherapy on survival rates (1,2). Studies in the non-Hodgkin's lymphomas showing a positive effect when adjuvant chemotherapy is added to radiotherapy for localized disease (3,4) are hard to interpret because of the absence of a group treated with chemotherapy alone; it may be that effective chemotherapy is all that is needed here as well. In tumors of Group B, however, adjuvant chemotherapy has been and continues to be a matter of intense interest and widespread clinical testing.

The second point has to do with the association between the effectiveness of a drug combination and the activity of its single-agent constituents. It seems safe to assert that demonstrably effective combinations are made from truly active single agents. Hodgkin's disease, testicular cancer, and the acute leukemias are curable with combinations that have been assembled from constituents with meaningful levels of activity. For the diseases in Group A, the best single agents (table 2) have high response rates and even rather respectable complete response rates. Although in most cases the durations of these responses are measured in months, durable remissions occur at low frequency. In contrast, the development of effective combinations for Group C has been consistently thwarted by lack of suitable building blocks; despite occasional claims to the contrary, combination treatment has not proved superior to single drugs, or for that matter to supportive care alone, in any disease of this group.

With respect to Group B, encompassing a broader spectrum of clinical behavior, meaningful generalizations are more difficult. On the whole, single-agent activity is intermediate between Groups A and C; partial response rates to "active" agents are generally between 10% and 40%, and complete response to single drugs is uncommon, even in the more sensitive tumors of the group.

With the wisdom of hindsight, then, we can say generally that the combinations have yielded more or less what we should have expected naively from the single agents used to build them. In no instance has a combination of relatively inactive agents converted a refractory solid tumor into a curable one. Progress in the treatment of disseminated disease has been substantial, but has been confined to those tumor types for which adequate tools exist; in those tumors, combinations dramatically increase survival.

During the period in which our present combinations were developed, the testing of drug combinations against transplantable murine tumor models proceeded with notable intensity. The preclinical and clinical activities moved along in parallel, each fertilizing and reinforcing the other. In the course of this effort, both groups of investigators have become more sophisticated in terms of the kinds of predictions one might reasonably expect

from murine models, given that human beings are not mice and autochthonous human tumors are not transplantable murine ones.

Before we consider what we have learned from the models and what we have failed to learn from them, we must first deal with certain areas in which the two cultures have been speaking different languages, or at least different dialects, and have been conducting their business according to quite different procedures.

## METHODS AND ENDPOINTS OF THERAPY EVALUATION

### Preclinical

Current techniques for the study of the effectiveness of drug combinations against model tumor systems evolved from earlier methodology in combination drug research (9,10). The initial medical interest in drug combinations sought improvement in therapeutic response. The general conceptual approach in support of combination chemotherapy hypothesized that two or more drugs employed together would allow a reduction in the dosage of each agent, thereby minimizing toxic side effects and providing a greater margin of safety. Combinations of drugs might also produce the desirable individual effects of each drug alone; for example, a combination might take advantage of the rapid action of one of the agents and the prolonged effect of the other.

The initial selection of mixtures of drugs for testing involved primarily empirical approaches, and no serious efforts were made at quantification of response. Nevertheless, the approaches and choice of model test systems emphasized the goal of obtaining improved therapies by employing combinations of drugs, ie, the attainment of "therapeutic synergism."

Following the initial clinical interest, combinations of chemicals, such as mixtures of insecticides or fungicides, were utilized increasingly in programs in which systemic toxicity or therapeutic response in man was not relevant. These developments stimulated evaluation of the mode of action of drug combinations. A variety of model systems was utilized to evaluate the extent of some designated biological response; improvement in therapy was not the goal at all. Frequently such programs attempted to decrease the amount of chemical agents required to obtain some desired objective, such as, for example, the destruction of a designated percentage of an insect population. Studies of this kind generally employed only a single parameter of response, such as the extent of toxicity or mortality. This contrasts with the problem of systemic therapy where one must consider the influence of treatment on both the host and the parasitic cells. Occasionally in the therapeutic situation reducing the amount of agent required to achieve the desired objective may be important, as in the days following the introduction of penicillin into clinical use. For the most part, however, drug supply is not a limiting feature in clinical medicine, at least not in economically developed countries.

A number of mathematical models have been developed for the investigation of the concerted action of drug combinations in nontherapeutic situations. The development of these models has been accompanied by the establishment of mathematical concepts pertaining to synergism, potentiation, and antagonism. Finney (11), for example, has a comprehensive mathematical discussion of the toxic actions of mixtures of poisons. Models that have been utilized to characterize the combined action of mixtures of poisons include: (*a*) "similar joint action," in which the modes of action of drugs employed in combination are similar; (*b*) "independent joint action," in which the individual components of the mixture act independently on the target organism; and (*c*) "synergism" and "antagonism," in which the potency of the mixture is more extensive or is less than would be expected relative to the potencies of the individual drugs, respectively (11,12). In the development of the mathematical approaches to synergism, which emphasized reduced quantities of chemical, there was still further drift in concept from the initial objective of improved therapy. Also, the mathematical approaches and methods established for these uses tended to be pressed into service for the investigation of synergism in all instances.

The introduction of antibiotics such as penicillin and the sulfonamides renewed interest in the therapeutic effectiveness of combinations of clinically useful drugs (13). In more recent years, the extensive emphasis on the improvement of antitumor effectiveness with drug combinations has led to broadening of the methodological approaches.

The diverse definitions of the terms synergism and antagonism are traceable to the varied interest of the investigators, as indicated above (9). Such definitions have included: (*a*) "cooperative action of discrete agencies such that the total effect is greater than the sum of the two effects taken independently—the opposite of antagonism" (14); (*b*) a quantitative change in the sense of "increasing the efficiency." The term "potentiated summation" has been used for the situation "where the combined action is greater . . . than could be calculated" (15); (*c*) "positive summation," the expression "potentiation" being retained for instances in which the combined action of two drugs is greater than that which would be expected from the sum of their individual actions (16); (*d*) "synergism implies the ability of two antimicrobial drugs acting together to increase markedly the rate of early bactericidal action, as compared to the rate with either drug alone, and to kill greater numbers of bacteria or to cure experimental or clinical infections more effectively than could be expected from simple algebraic summation of single drug effects" (13); and (*e*) "the combination effects a certain response with a smaller number of molecules than that required for the most active com-

pound separately, or: in the range of suboptimal concentrations, the effect of a certain number of molecules of this compound is enhanced in the mixture" (17).

In the case of antineoplastic chemotherapy, synergism has usually referred to the cooperation or effective combination of agents in the achievement of an improved therapy (18) compared with the drugs employed individually. Goldin et al (19) used this concept of synergism effectively and it was extensively applied in subsequent investigations.

The quantitative evaluation of drug effect in the laboratory is reasonably straightforward. Typically, as in the leukemia L1210 dose-survival time system, a dose-response curve is established. With an active therapeutic agent such as methotrexate, as the dosage is increased the survival time of the tumor-bearing animals is extended until a maximum therapeutic effect is reached at the optimal dosage (19). As the dosage of the agent is increased further, the toxicity of the drug is manifested and the survival time of the animals diminishes. This type of system provides a quantitative measure of drug effectiveness and permits evaluation of antitumor efficacy on the basis of the optimal increase in survival time or the number of remissions of tumor, duration of remission, or number of cures, and also on the basis of the dosage range over which a particular drug is effective. The maximum increase in survival time at the optimal dosage is a measure of therapeutic effectiveness of a drug. The width of the curve is an indication of the degree of safety with which the drug can be employed. In this system, the therapeutic index or ratio may be represented as the ratio of the optimal dose to the dosage which results in a specified increase in survival time of the animals (20).

The dose survival-time response system can be employed for the quantitative comparison of the therapeutic efficacy of drugs. In an early study (21), methotrexate appeared approximately twice as effective as either 6-thioguanine or 6-mercaptopurine in increasing the survival time of mice with advanced leukemia L1210. Six-thioguanine and 6-mercaptopurine were about equally active in prolonging the life span of tumor-bearing mice. Azaserine was about one-half as effective as 6-thioguanine and 6-mercaptopurine. Interestingly, 6-mercaptopurine and 6-thioguanine were equal in therapeutic activity, even though the effective dose range for the two drugs was considerably different. Thus, this methodology makes it possible to rank the effects of drugs on the survival time of tumor-bearing animals. These considerations have had an important bearing on the selection of drugs for clinical trial.

This procedure obviously lends itself to a practical determination of the presence of therapeutic synergism when drugs are used in combination. Specifically, one establishes dose survival-time response curves for the drugs individually and in combination. One then compares the maximum increase in survival time at optimal doses for the drugs individually and the combinations. Synergism then exists if the combination produces an increase in survival time greater than that obtained with the drugs individually at their optimal doses. As described earlier for single agents, the dose-survival time system permits the characterization and ranking of the effectiveness of combinations of drugs relative to the drugs employed individually and relative to other combinations.

An essential feature of such an evaluation of new combinations is a systematic study of the effects of different drug schedules and dosage ratios. Much evidence attests to the often significant effects of changes in either of these parameters on treatment effectiveness. The data with carmustine and methotrexate (22) in table 3, for example, are typical of results with many drug combinatons in the dependence of therapeutic effect on dose ratio. Note that optimal dose levels are listed at each of the dosage ratios. In experiments with 5-FU plus methotrexate (96), variations in dose ratio from 4:1 to 8:1 resulted in a 50% increase in median survival time from 21 to 31.5 days. Similar experiments with mitoguazone and 6-mercaptopurine documented an increase of about 30% in median survival time when the dose ratio varied from 1:4 to 1:2 (26). In a particularly dramatic example, in-

TABLE 3.—Relative effectiveness of carmustine and methotrexate, separately and in combination, in the daily treatment of advanced leukemia L1210*

| Mg/kg dosage ratio | Optimal treatment levels | | Median survival time (days) | Increase in median survival time over controls (%) |
|---|---|---|---|---|
| | Carmustine | Methotrexate | | |
| 4:1 | 3.0 | 0.75 | 62 | 464 |
| 2:1 | 3.0 | 1.5 | 55.5 | 405 |
| 1:1 | 1.5 | 1.5 | 38 | 245 |
| 1:2 | 0.75 | 1.5 | 31.5 | 186 |
| 1:8 | 0.09 | 0.75 | 27 | 145 |
| — | 6.0 | 0 | 41.5 | 277 |
| — | 0 | 0.75 | 27.5 | 150 |
| Controls | | | 11 | |

*Treatment was begun 7 days after sc leukemia inoculation and continued until death. There were 10 $BDF_1$ male mice/group and 20 untreated controls (*modified from* ref 22).

crease in life span values for the optimal dose levels of the combination of razoxane and camptothecin against early L1210 leukemia varied over a more than fourfold range; increases in life span values were 133% at a 20:1 ratio, 411% at 10:1, and 560% at 5:1 (22).

Scheduling may also exert a profound effect on therapeutic effectiveness with combinations as it does with single agents. Choice of optimal scheduling may greatly enhance antitumor effectiveness. In a notable example with a single agent, intermittent (every-4-day) treatment of early leukemia L1210 with methotrexate was considerably more effective than daily treatment in increasing survival time (23). This observation appeared to correlate with clinical results, which showed that biweekly treatment was more effective than daily treatment in remission maintenance of children with acute leukemia (24).

A classic example of the schedule dependency of therapeutic synergism with combinations of drugs is provided by the combination of 6-mercaptopurine plus azaserine in the treatment of leukemia L1210; with treatment every 2 days, 6-mercaptopurine plus azaserine evidenced therapeutic synergism, being more effective than 6-mercaptopurine alone or azaserine alone (25). On daily treatment, therapeutic synergism was less evident. When the drugs were administered twice daily, no therapeutic synergism was observed, and the combination was no more effective than the drugs employed individually.

Another example of schedule dependency with combination chemotherapy is presented in table 4 (26). Treatment with methotrexate given daily plus cyclophosphamide weekly was more effective than methotrexate daily plus cyclophosphamide daily, and more effective than the drugs employed individually in increasing the survival time of leukemic animals. When razoxane and cisplatin were administered every 4 days (Days 1, 5, and 9), the combination was considerably more effective than either drug alone in increasing the survival time of mice with leukemia L1210. However, when cisplatin was given on Days 1, 5, and 9 and razoxane was given every 3 hours on Days 1, 5, and 9 (an optimal schedule for the latter drug individually), the combination did not exhibit synergism.

Experience with methotrexate and L-asparaginase in leukemia L5178Y indicates (27) that the sequence of administration of two drugs may alter the therapeutic response (table 5). In the first sequence, methotrexate was given on Days 3–7, followed by L-asparaginase on Days 10–14, and a marked therapeutic response was observed. But when L-asparaginase was administered on Days 3–7 and followed by methotrexate on Days 10–14, no therapeutic advantage was observed. Systematic study has indicated that the sequence of administration of cyclophosphamide and methotrexate may have an important effect on antileukemic response (28). Similarly, Tattersall et al (29) have noted the importance of the sequence of administration of methotrexate and cytarabine in a mouse lymphoma system, in which the drugs were most effective when given simultaneously. Cytarabine given 6 hours before methotrexate gave a result similar to a single agent, while methotrexate given 6 hours before cytarabine produced an overall antagonistic effect.

Obviously, then, the complete evaluation of a particular combination of two drugs may be a very laborious procedure and implies systematic determination of the effects of alterations in scheduling, dose ratio, and dose intensity on therapeutic efficacy.

## Clinical

The clinical development of active combinations departs substantially in methodology from the preclinical paradigm. In the first place, clinicians generally use the term synergism very loosely; authors often simply state that promising results with a particular combination (ie, higher response rates than one might vaguely expect

TABLE 4.—Influence of treatment schedule on effectiveness of methotrexate (MTX) and cyclophosphamide (CPA) alone and in combination against advanced leukemia L1210*

| Treatment | Optimal dose (mg/kg) | Median survival time (days) | Ratio of median survival time of indicated treatment to that of daily MTX |
|---|---|---|---|
| MTX alone (daily) | 1.08 | 29 | 1.00 |
| CPA alone (daily) | 65.0 | 27.5 | .92 |
| MTX (daily) | 0.65 | 31 | 1.11 |
| + CPA (daily) | + 39.0 | | |
| MTX alone (daily) | 1.08 | 26 | 1.00 |
| CPA alone (daily) | 39.0 | 26 | 1.00 |
| CPA alone (weekly) | 180 | 27.5 | 1.10 |
| MTX (daily) | 1.10 | 39 | 1.87 |
| + CPA (weekly) | + 108 | | |
| Controls | — | 11 | — |

*Sc treatment was begun 8 days after sc tumor inoculation and continued daily until death. The treatment level shown for each therapy was the most effective among the wide range of levels employed. *Data from* ref 26.

TABLE 5.—Schedule dependency of combination treatment with L-asparaginase (L-Ase) and methotrexate (MTX) in leukemic (L5178Y) mice *

| Treatment schedule | | | Dose/day | | Median survival time (days) | % increase over controls |
|---|---|---|---|---|---|---|
| Days 3-7 | | Days 10-14 | Days 3-7 | Days 10-14 | | |
| MTX | | | 3 mg/kg | | 21 | 40 |
| | | MTX | | 3 mg/kg | 19.5 | 30 |
| L-Ase | | | 23 IU | | 19 | 26.7 |
| | | L-Ase | | 23 IU | 19 | 26.7 |
| MTX | + | L-Ase | 3 mg/kg | 23 IU | 32 | 100 |
| L-Ase | + | MTX | 23 IU | 3 mg/kg | 16 | 7 |
| Untreated controls | | | | | 15 | — |

* Treatment given ip; L5178Y $10^6$ cells given sc. $CDF_1$ mice used. *Data from* ref 27.

from the single agents) suggest synergism, without realizing that the term should be a quantitative description of drug effect needing a sensible and precise definition.

In seeking a useful definition for clinical use, the most satisfying possibility would be to define synergism in a manner parallel to laboratory usage; the term would then refer to combinations which give objectively improved therapy compared with the components. It would not refer to nonselective enhancement of cytotoxicity or to biochemical interactions. By analogy with the laboratory, the demonstration of therapeutic synergism in the clinic would then require first the definition of an optimal dose for the constituent agents singly, then the exploration of the new combination in one or more schedules, each including a range of dose ratios and dose intensities. Combinations which yield better therapy than the single agents used optimally may then be rigorously termed synergistic.

The problems with this approach are obvious. First of all, clinicians may speak of the maximally tolerated dose (MTD) or the recommended phase II dose, but they rarely speak of the optimal dose of a drug. To the extent that clinical dose-response curves increase monotonically, it might be reasonable to assume that the MTD is an optimal dose, at least in terms of tumor shrinkage. However, clinicians have never been fond of formal dose-response evaluations, and the relation of single-agent dose to survival must usually remain conjectural. In fact, since most single drugs do not appear to have profound effects on survival for most tumors in phase II trials, one might anticipate that survival comparisons would not yield much information in the context of a dose-response study.

Second, the existence of modern techniques in supportive care has permitted dose escalation to levels far above those that would be maximal in the absence of intensive support. Broad-spectrum antibiotics and blood products, to say nothing of bone marrow transplantation, have operationally redefined the meaning of MTD. In the clinic, the very term MTD is only meaningful in the context of a clear definition of the level of toxicity one is willing to tolerate. In this sense, the clinical MTD is a more relative concept than the experimentalist's optimal dose or lethal dose levels, which are defined in the context of standardized laboratory techniques.

Finally, if clinicians did attempt to determine unambiguously the dose-response or dose-survival relationships with single drugs, the requirements in terms of time and patient numbers would be very great. They would become astronomical if combinations, even simple two-drug combinations, were tested in the clinic in a manner at all analogous to laboratory practice.

However impossible the direct translation of the laboratory paradigm to the clinic may be, recognition of the differences serves to suggest how incomplete we are in the formal evaluation of clinical therapies, whether single drugs or combinations. It also emphasizes that we have no firm basis on which to evaluate quantitatively the predictive value of preclinical findings with drug combinations.

Another troublesome difference between laboratory and clinic relates to the stage of disease at the time of the principal therapeutic trial. In models, much work is carried out at minimal tumor burden; therapy is begun within a day or two of tumor inoculation. By contrast, the clinical testing of new combinations is almost invariably carried out in patients with advanced measurable disease. Only after a regimen shows substantial activity in this setting is its use against minimum residual disease felt to be justifiable or ethical. In short, combinations may be recommended for clinical consideration based on impressive experimental results in early disease with survival prolongation as the chief (sometimes the only) measure of efficacy. Clinicians may then proceed to evaluate the regimen in late disease with tumor shrinkage as the principal measure of success.

Is this a reasonable procedure? More specifically, might a combination be significantly better than its constituents individually at low tumor burdens, but no better at high burdens? A dependence of effect on body burden is commonly seen when single agents are tested in preclinical models (157). Similarly with combinations, loss of therapeutic synergism may also occur as tumor burden increases; for example, the combination of dacarbazine plus 5-FU, which shows therapeutic synergism against

early leukemia L1210, loses much of its advantage over the single agents against more advanced disease (table 6) (30). Whether a similar dependence exists in human disease is unclear, since the relevant data sets are not available for comparison; one would need results from paired sets of clinical trials in which a regimen was compared with the suitable single-drug controls in both the adjuvant and advanced disease settings. Application of the somatic mutation theory, however, suggests that dependence of therapeutic synergism on tumor burden might be important indeed, since the prevalence of multidrug resistance ought to increase sharply as a function of tumor load.

Many investigators have acknowledged the substantial biological differences between transplantable murine tumors and spontaneous human cancer (31). The model tumors are apparently less heterogeneous, are more active kinetically (32), have less cell loss during growth (32), and are far more susceptible to killing by present-day cytotoxic agents than are most human tumors (33). The extent to which specific findings in transplantable models apply to human cancer will continue to be a major problem, and we agree with Martin et al (34) that more studies ought to be done in spontaneous models. Many investigators have emphasized that no murine tumor is a precise model for any specific human tumor; this requires re-emphasis as superficial similarities between certain murine cancers and their clinical counterparts have stimulated suggestions for specific clinical trials (33).

With these differences in mind, let us look at the relation between combination chemotherapy in the laboratory and the clinic.

## RATIONALE FOR SELECTION OF COMBINATIONS

When two cytotoxic drugs are administered in combination, they may exert their cytotoxic effects independently or they may interact in some pharmacological or biochemical manner. Consider first the possibility of independent actions. Drugs A and B are not cross-resistant, neither affects the other's pharmacokinetics or pharmacodynamics, and each kills its respectively sensitive subpopulations independently. In such circumstances, we should expect the net effect to be a combination of the independent effects of each. In particular, if we are treating hosts with measurable disease, we might postulate a model in which the probability $R_{AB}$ of responding to the combination AB is related to the probability of responding to the individual agents $R_A$ and $R_B$ as follows:

$$R_{AB} = 1 - [(1 - R_A)(1 - R_B)]$$

provided that A and B can both be given at full dose in the combination. More generally, for any number of drugs $D_1 \ldots D_N$,

$$R_{D_1 \cdots D_N} = 1 - \left[\prod_{i=1}^{N} (1-R_i)\right]$$

Satisfaction of this relationship ought to be a necessary condition for independence, although by no means a sufficient one.

Interestingly, if we use conventionally determined clinical response rates as estimates of these probabilities, this relationship appears to be satisfied by a number of combinations studied in the clinic (table 7). In evaluating agents for independence in this way, the response rates for the single agents and the combination ought to be derived from prognostically similar patient populations. In addition, single-agent doses should be closely similar to corresponding doses in the combination. Finally, response criteria should be similar for the single agents and the combination; this may pose a significant problem if one attempts to use data from different time periods. As shown in table 7, the rather close agreement between the calculated and actual response data in a few familiar clinical examples is compatible with a hypothesis of independent action in these cases.

In contrast, two drugs may jointly cause damage within the same cellular targets. If they do this in ways which enhance cytotoxicity compared to the component agents used singly, therapeutic synergism may result. Synergism is by no means guaranteed, however, since enhanced toxicity to normal host cells may also occur, yielding no net increase in therapeutic effect. If synergism does occur, the reason is that, unlike the case of independent action outlined above, the combination induces greater cell kill in the *same* malignant cell targets than each agent individually, without simultaneous increase in normal tissue damage. This scheme is distinct from biochemical modulation, in which one agent is used in a noncytotoxic manner to enhance the cytotoxic effects of a second drug.

The neat division between independent and joint drug action is likely to be a significant oversimplification of what occurs in vivo. In view of the heterogeneity of most cancers, a pair of agents capable of interacting with each other to produce enhanced killing may well have particu-

TABLE 6.—Influence of day of treatment initiation on response of leukemic (L1210) mice to dacarbazine (DTIC) and 5-FU, separately or in combination*

| Treatment | Optimal dose (mg/kg/day) | Increase in life span over controls (%) |
|---|---|---|
| *Treatment daily from Days 1 to 18* | | |
| 5-FU alone | 14 | 95 |
| DTIC alone | 300 | 67 |
| 5-FU + DTIC | 23 + 65 | 250 |
| *Treatment daily from Days 5 to 18* | | |
| 5-FU alone | 14 | 100 |
| DTIC alone | 300 | 20 |
| 5-FU + DTIC | 8.4 + 23 | 144 |

* *Data from* ref 30.

TABLE 7.—Comparison of observed response rates to combinations with calculated rates based on response rates of single agents under assumption of independent action*

| Disease† | Drugs | Single-agent response rates | Combination response rates | |
|---|---|---|---|---|
| | | | Calculated | Observed |
| Lung, NSC | Cisplatin (120 mg/m²) | .26 | .42 | .43 |
| | Vindesine | .21 | | |
| Lung, NSC | Cisplatin (< 120 mg/m²) | .19 | .34 | .37 |
| | Etoposide | .18 | | |
| ANLL | Cytarabine (100 mg/m²/day to hypoplasia) | .14 | .55 | .58 |
| | Daunorubicin (60 mg/m²/day × 3) | .48 | | |
| Hodgkin's disease | Mechlorethamine | .63 (.13) | .96 (.65) | 1.00 (.40-.80) |
| | Vincristine | .60 (.36) | | |
| | Procarbazine | .69 (.38) | | |
| | Prednisone | .28 (0) | | |

* Response rate data for non-small cell lung cancer *from* ref 35, for ANLL *from* refs 36 and 37, and for Hodgkin's disease *from* refs 38 and 39. For Hodgkin's disease, values in parentheses are complete response rates. For ANLL, values are complete response rates.
†NSC = non-small cell; ANLL = acute nonlymphoblastic leukemia.

lar lethality for cellular targets sensitive to only one or the other. The net result may be a multiplicity of lethal events which would prove difficult or impossible to sort out unambiguously.

Bearing in mind these rather simplistic considerations, we now discuss the rationales for the construction of combinations in terms of a few broad operational classes.

### Combinations Based on Single-Agent Activity

The hypothesis here is that combinations of agents which are themselves active individually may be more active than their constituents used singly. In the laboratory, such combinations have commonly been employed in attempts to demonstrate therapeutic synergism. (22,26,33,40-78) (table 8). Data illustrating the synergism observed with carmustine and methotrexate against L1210 leukemia are shown in table 3 (22).

In view of the apparent ease with which synergistic and very effective combinations against transplantable murine tumors can be constructed, it is worth re-emphasizing that they are made of very effective building blocks. The available single agents have very impressive total (and in many cases complete) response rates against murine models even at high tumor burdens (33); positive effects on survival are easily discernible with most single agents and cures are also attainable.

The empirical formation of combinations from putatively active single agents has traditionally been the most widely used procedure clinically; despite increasing scientific sophistication, the construction of regimens still relies heavily on empirical considerations. In 1984, a total of 60 single-arm studies of drug combinations appeared in *Cancer Treatment Reports, Cancer,* and the *Journal of Clinical Oncology.* Of these, 42 were motivated by purely clinical considerations. As shown in table 9, clinical activity of single agents was by far the most frequently cited specific rationale for performing the trial. In fact, this is even a more prevalent motivation than the figures in table 9 suggest, because many of the other categories represent continued study of combinations originally brought to clinical trial because of single-agent activity of the components. This finding is, of course, no surprise; building regimens in this way is fairly easy, and, when done intelligently, the empirical approach has produced some stunning successes.

On the whole, however, this process has produced far more failures than successes. There are at least four important reasons for this. First, our criteria for what constitutes sufficient "activity" to justify incorporation into combinations have perhaps been unrealistically low; generally, agents with 10%-20% partial response rates seem to contribute little to combinations other than toxicity.

Second, many investigators have paid altogether too little attention to the relation of dose to response; because of the overlapping toxic effects of many cytotoxic agents, their use in combination is often accompanied by a significant reduction from the full single-agent dose. One can hardly expect a drug with a 40% response rate to contribute this level of activity to a combination if its dose intensity within that combination is significantly less than the single-agent dose intensity that yields the 40% response rate.

Third, agents have generally been placed into combina-

TABLE 8.—Selected examples of therapeutic synergism against transplantable tumors*

| Drugs† | Tumor | Stage |
|---|---|---|
| 5-FU + cisplatin | L1210 | Intermediate |
| CPA + dacarbazine | L1210 | Early |
| CPA + nitrosoureas | L1210 | Early |
| CPA + carmustine | KHT fibrosarcoma | Advanced |
| | AKR leukemia | Advanced |
| | L1210 | Early |
| | C3H mammary | Early |
| CPA + semustine | AKR leukemia (spontaneous) | Advanced |
| | Glioma 26 | Advanced |
| | Adenocarcinoma 755 | Early |
| | C3H mammary | Early |
| | B16 melanoma | Early |
| | Sarcoma 180 | Early |
| | Lewis lung | Advanced |
| CPA + melphalan | L1210 | Advanced |
| | P388 | Early |
| | Ridgway osteogenic sarcoma | Advanced |
| | B16 melanoma | Early |
| CPA + cytarabine | L1210 | Early |
| CPA + cisplatin | L1210 | Intermediate |
| DTIC + Dox | L1210 | Early |
| Ifos + cisplatin | L1210 | Intermediate |
| Dox + 5-FU | Mammary 16/C, 04/A, spontaneous $CD8F_1$ | Advanced |
| Dox + CPA | C3H mammary | Early |
| | P388 | — |
| | B16 melanoma | Early |
| | Ridgway osteogenic sarcoma | Advanced |

* *Data from* refs 22, 57, and 83.

† CPA = cyclophosphamide; DTIC = dacarbazine; Dox = doxorubicin; and Ifos = ifosfamide.

TABLE 9.—Rationales for clinical studies of drug combinations appearing in 1984

| Rationale | No. of studies |
|---|---|
| Clinical studies | 42 |
| Clinical activity of single agents | 17 |
| Confirmation of previously reported results | 8 |
| Drug substitution to decrease toxicity | 4 |
| Active combination extended to another tumor | 3 |
| Follow-up of preliminary report | 2 |
| Active combination extended to other stages | 1 |
| Dose or schedule modification of previous regimen | 1 |
| Pharmacologic considerations | 1 |
| Alternating combinations | 1 |
| No clear reason given | 4 |
| Preclinical studies with or without clinical | 18* |
| Sequential methotrexate-5-FU | 7 |
| Other findings of synergy | 4 |
| Other biomedical modulation | 2 |
| Drug access | 7 |
| Total | 60 |

* Several of these studies had multiple rationales given.

tions without much regard for patterns of cross-resistance with other agents in the combination. This is admittedly a very complex area whose subtleties are only beginning to come to light. Recent advances in the characterization of the pleiotropic drug resistance phenotype by Ling et al (80) have shown that resistance to multiple agents can be acquired simultaneously by a single genetic event. Multidrug resistance appears to exist in human tumors as well (133), but the patterns of cross-resistance are heterogeneous and different from those observed in nonhuman cell lines. Although the prevalence of specific phenotypes in human tumors is unknown, it is perfectly plain that this existence of acquired or de novo multidrug resistance is a major obstacle to the construction of effective combinations. This phenomenon may help explain why combinations containing several natural product derivatives have failed to induce high rates of durable complete remissions in some diseases; regimens for small cell lung cancer, for example, commonly contain vincristine, doxorubicin, and etoposide.

Finally, the methodology used in many exploratory studies of new combinations (ie, a straight phase II approach with small numbers of patients and without an explicit control group or an analysis adjusting for differences in prognostic factors) is probably an unreliable technique for revealing any but the most dramatic differences from expected results. When investigators perform trials of this kind, they are particularly interested in remission rates (total and complete), duration of remission and survival, and toxicity. Each of these endpoints can only really be evaluated by comparison with pertinent alternative therapies; unless the regimen is utterly inactive or horrendously toxic, questions involving comparison with previously studied regimens naturally arise. Perusal of the 60 uncontrolled studies of combinations published in 1984 confirm that investigators commonly make statements about efficacy (is the treatment medically worthwhile?) rather than simply activity (does the treatment cause shrinkage of tumor?), despite the absence of a suitable comparison group. Moreover, the sample sizes of at least 22 of these studies were so small that confidence intervals around the response rates failed to distinguish between active and inactive.

Is the empirical finding of therapeutic synergism in the laboratory a reliable predictor of potential clinical synergism? It is difficult even to approach this question, since as noted previously, clinical synergism has never been precisely defined and the relevant clinical data sets do not exist. Certainly, the preclinical finding of therapeutic synergism has motivated clinical trials of particular combinations. The oncologist examining table 8 will recognize many combinations which have been in clinical trial or have become incorporated into conventional regimens of a particular disease. Unquestionably, many two-drug combinations that exhibit therapeutic synergism against transplantable murine tumor models have shown some degree of clinical usefulness (although this is by no means invariably the case). Clearly, laboratory data have been useful in motivating the testing of particular combina-

tions, even if the clinical realities noted previously make the real answer to the existence of synergism in human cancer elusive.

It is also admittedly not clear whether the preclinical finding of synergism adds substantially to an approach which would reserve for clinical development in combinations only those drugs with solid clinical credentials as single agents. Experience with two combinations of current interest illustrate the issues well. Nearly 10 years ago the combination of cisplatin and etoposide was introduced into clinical trial against small cell lung cancer (SCLC) (81). The rationale was empirical in the extreme. Etoposide was known to be probably the most active single agent against SCLC. Although no information was available on cisplatin's activity, the drug had a broad spectrum in human malignancy and we strongly suspected it would also be active against this sensitive tumor. In fact, we feared that a negative phase II study of cisplatin, performed in heavily pretreated patients by necessity, would more likely represent a false than a true negative result, or at the very least would significantly underestimate the potential value of cisplatin in previously untreated cases.

In any case, the combination of cisplatin and etoposide turned out to be highly active against SCLC. After the clinical trial was well under way, impressive degrees of synergism were reported against P388 leukemia (74,78), B16 melanoma (75), and the FANFT-induced bladder carcinoma (82). The existence of preclinical synergism together with the early clinical results in SCLC stimulated clinical testing in additional tumor types. The combination has turned out to be curative in germ cell tumors of the testis (2) and active in the lymphomas and non-small cell lung cancer. In addition to further studies in these diseases, trials are currently in progress against carcinomas of breast and bladder.

The experience with this regimen certainly shows how clinical and preclinical data can be mutually reinforcing in stimulating clinical trials. Although the high complete response rate in previously untreated patients with SCLC seems greater than one might expect from the activities of the two agents singly, there are really insufficient single-agent data in previously untreated patients to be at all certain. It therefore remains unclear whether the effect of the combination is compatible with independent action of the components or whether the results suggest interaction. In this respect, results of the breast and bladder trials will be of interest. In breast cancer, etoposide is essentially inactive, as is cisplatin in doses < 100-120 mg/m$^2$. In bladder cancer, etoposide has a low level of activity. If the two agents act independently, one would predict that the combination would be inactive in breast cancer and about equal to cisplatin alone in bladder cancer.

The combination of cisplatin and 5-FU has attracted great interest recently because of high activity against squamous head and neck cancer (158). The initial trial was motivated by evidence of preclinical synergism (159), by the individual activity of the two agents in head and neck cancer, and by the desire to find a less toxic substitute for bleomycin. 5-FU was given by continuous iv infusion, a schedule for which there are no reliable single-agent data in head and neck cancer. Activity of the combination has been striking, and the regimen is currently under test as an adjuvant in multicenter trials. Again, as with cisplatin and etoposide, because of lack of comparable single-agent data, it is impossible to estimate whether the high response rates are explicable on the basis of independent action or not. Surprisingly, the combination has also shown interesting levels of activity against colorectal carcinoma (160), in which cisplatin alone is quite inactive. In view of the enormous range of response rates reported for fluoropyrimidines alone against colorectal carcinoma, however, and the failure of at least one group to confirm these results (161), the data with cisplatin and 5-FU are impossible to interpret. Fortunately, controlled trials are now in progress.

In summary, a huge amount of clinical testing of combinations based largely on empirical considerations has taken place in the common solid tumors. Much of this effort has been motivated by considerations of clinical single-agent activity. Despite occasional successes, and a less impressive but still significant increase in antitumor activity against certain relatively sensitive tumor types such as the carcinomas of breast, ovary, head and neck, and bladder, the development of curative combinations for most categories of disseminated disease still lies in the future. From our successes in both laboratory and clinic, it seems clear that the purely empirical construction of combinations is a high-yield procedure only when truly active agents are available. Viewed in this way, a major obstacle to the development of effective combinations is simply the lack of really active single agents.

## Combinations Based on Mechanistic Hypotheses

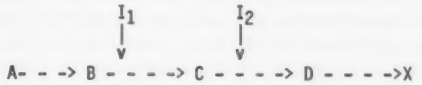
A variety of features pertaining to target tumor cells may increase the therapeutic action of anticancer drugs (22,41,50). These may include, for example, decrease in a critical metabolite, precursor, or product of enzymatic transformation; decreased activity of an essential enzyme; or increase in concentration or time of exposure to an antagonist. Actions of two or more agents which tend to increase the extent of inhibition of vital intracellular processes may lead to improved therapy, provided that the inhibition exerts selectivity against tumor cells.

### *Multiple Biochemical Blockade*

Many varieties of cooperative interference with biochemical pathways have been proposed as explanations for the enhanced cytocidal effects of certain combinations (50).

*Sequential blockade.*—Potter (84) developed the con-

cept of sequential blockade, where two inhibitors exert their action at sequential loci in a series of metabolic transformations.




In this system, if there is inhibition, for example, of the transformation B- - -> C by inhibitor $I_1$, this would tend to reduce the concentration of the metabolite C and thereby render the enzyme concerned with the conversion of C to D more sensitive to the inhibitory action of inhibitor $I_2$. The resultant synergism pertaining to a parameter such as cell death involves an increase in the inhibitory effect on a molar basis and it would be possible to achieve a desired effect with a smaller total amount of drug. In this system, it may be possible to achieve the same level of inhibition obtained with the combination of inhibitors by merely increasing the concentration of each of the inhibitors individually. In the therapeutic situation, however, where toxicity of the drug for the host is limiting, there might be an advantage in employing two drugs at lower concentrations to achieve a greater antitumor effect, provided there is not a proportionate increase in toxicity for the host. This example illustrates the important difference between simple synergism at the level of cell killing and therapeutic synergism.

The combination of cytarabine, which inhibits DNA polymerase after conversion to Ara-CTP (85), with guanazole, hydroxyurea, 5-hydroxypicolinaldehyde thiosemicarbazone, or picolinaldehyde thiosemicarbazone, which are all inhibitors of ribonucleotide reductase (86-89), provides examples of sequential inhibition of the synthesis of DNA; these combinations have been demonstrated to provide therapeutic synergism in the leukemia L1210 system (22,40,88,90-92).

The enzymatic inhibitory actions of methotrexate and 5-FU were originally viewed as a form of sequential blockade. Inhibition of dihydrofolate reductase by methotrexate prevents the formation of tetrahydrofolate from dihydrofolate (93), and inhibition of thymidylate synthetase by FdUMP diminishes the transformation of 5-10-methylene tetrahydrofolate to dihydrofolate (94,95). Despite the demonstration of therapeutic synergism with this combination (96), however, it is not clear whether this biochemical rationale is indeed the underlying mechanism for the observed synergism. In other investigations (97-99), synergy was found when methotrexate preceded 5-FU in vitro and in vivo, and compelling alternative mechanisms have been proposed. This combination is currently the subject of great interest clinically (100).

A modified form of this type of inhibition perhaps occurs with certain single agents which apparently act at several loci in a metabolic sequence; one agent serves as both $I_1$ and $I_2$. Antifols, for example, primarily inhibit dihydrofolate reductase, but also may under proper conditions inhibit thymidylate synthetase (101). Treatment with 6-thioguanine alone inhibits enzymatic transformation at two points in purine nucleotide biosynthesis and may therefore provide an example of sequential blockade induced by a single agent. Following conversion to the active nucleotide, 6-thioguanine inhibits the enzymatic activity of phosphorybosylpyrophosphate amidotransferase, inosine 5′-phosphate dehydrogenase, and ATP:GMP phosphotransferase. This form of sequential blockade may result in a decrease of guanine nucleotides in the cells with a resultant decrease in the rate of synthesis of RNA and DNA and a decrease in activities of a number of enzymatic reactions in which GTP is involved as a coenzyme (102).

*Concurrent blockade.*—Elion et al (103) stressed the concept of concurrent blockade in which two inhibitors act at sites in alternate pathways that are involved in the synthesis of a common product. For this type of multiple biochemical blockade, knowledge may be utilized of the sequence of metabolic transformations in the biosynthesis of purine nucleotides for the investigation of the predictability of synergism (9,101-104).

*Complementary inhibition.*—Here the utilization of two or more agents results in damage to selected macromolecular targets (41-43). One of the drugs inhibits the biosynthetic pathway to a macromolecular target and/or repair and the other directly damages the already synthesized macromolecule itself. There are a number of examples of complementary inhibition in which the macromolecular structure under attack has been either DNA, RNA, or protein (41). In general, alkylating agents have been utilized to damage the DNA template, but intercalators such as the acridines may also be useful in combination with agents that interfere with the formation and utilization of deoxyribonucleoside triphosphate precursors of DNA.

The antibiotic mitomycin, which is capable of cross-linking complementary strands of the DNA double helix, as well as uracil mustard, carmustine, and lomustine, has been demonstrated to yield synergistic inhibition of tumor growth in combination with various antimetabolites that are capable of lowering intracellular deoxyribonucleoside triphosphate pools (41).

An example in which complementary inhibition has been directed against RNA involves the combination of dactinomycin and ribonuclease in synergistic inhibition of growth of the transplantable tumor Sarcoma 180 (105). It was considered that dactinomycin would function in the combination by inhibiting the synthesis of new RNA and that the ribonuclease would destroy preexisting molecules of RNA. Indeed, it was observed that the total cellular content of RNA of tumor cells treated with this combination of agents was markedly reduced.

An example in which complementary inhibition involves the synthesis of protein is provided by the combination of L-asparaginase and the glutamine antagonist 6-

diazo-5-oxo-L-norleucine (DON) in the treatment of animal tumors (106). With this combination, synergistic antitumor activity would result from the L-asparaginase limiting the external source of asparagine to neoplastic cells by a reduction in blood levels of asparaginase, and the antagonist DON would appear to act by direct inhibition of asparagine synthetase present either in tumor cells or in normal cells, which could serve to supply asparagine to the malignant cells. Another example is the antitumor synergistic action of the combination of L-asparaginase and 5-chloro-4-oxo-2-aminopentanoic acid (108), a potent inhibitor of asparagine synthetase (107). With these combinations, there may be a reduction in the asparagine pool in the neoplastic cells.

*Error amplification.*—Error amplification provides another example of a biochemical mechanism that may result in therapeutic synergism (41). In this approach, an attempt is made to induce transcriptional and translational errors resulting from point mutations or frame shifts. Agents such as ethyl methanesulfonate and *N*-methyl-*N'*-nitroso-*N*-nitroguanidine may produce point mutations, and the acridines may yield frame shift mutations. In error amplification, genomic lesions could be magnified by an agent such as 5-fluouro-2'-deoxyuridine which might produce a deficiency of thymine deoxyribonucleotides, thus inhibiting repair while synthesis of RNA and protein continues. This imbalance could result in the formation of multiple copies of missense or nonsense messenger RNA.

*Single-enzyme inhibition.*—In this case, binding of one drug to the enzyme tends to increase the binding affinity of the other drugs (108,109).

$$EI_1 + I_2 - - -> EI_1I_2 \quad (I_1 \text{ enhances binding of } I_2)$$

*Concerted inhibition.*—There is inhibition of two sites (110-112) within a regulated metabolic framework.

*Enhanced activation.*—The action of one drug favors the activation of the second drug (110-112).

*Decreased inactivation.*—Application of one drug inhibits the degradation of the other drug (113).

*Increased levels.*—The first drug may increase the cellular or organelle levels of the second drug. For example, vincristine appears to augment the steady-state level of methotrexate in L1210 tumor cells in vitro (114).

#### *Kinetic Synchronization*

Tumor cell synchronization may also be employed to therapeutic advantage with the use of a combination of drugs. Attempts to exploit the kinetic properties of tumors have motivated many experimental and clinical studies (115,116).

Attempts have been made to synchronize the tumor cell population with one drug and to then treat with a second drug at a time when the tumor cell population is synchronously traversing a particular phase of the cell cycle at which time it will be sensitive to the second drug. Vinblastine and demecolcine were employed to synchronize leukemia L1210 cells before treatment with cytarabine. When cytarabine was administered at the time that the synchronized tumor cell population had reached S-phase, a distinct therapeutic advantage was observed (117).

Consideration of cell kinetics has served as a basis for the selection of various experimental drug combinations. Agents that act at different stages of the cell cycle, and compounds with different mechanisms of action, have exhibited enhanced antitumor effects when given together. Examples of such drug combinations are cytarabine plus etoposide or teniposide (118).

Frankfurt (119) injected cyclophosphamide 6 hours after vinblastine and produced a greater cell kill than that observed with simultaneous injections of both agents. The synergism was probably related to the disappearance of cells in phase $G_0$ which are somewhat resistant to cyclophosphamide. In studies with a rat neurosarcoma, on the other hand, Volm and Wayss (120) reported that partial synchronization with hydroxyurea did not augment the antitumor effect of cyclophosphamide or doxorubicin.

In a variation of this approach, particularly for solid tumors where there is a large $G_0$ resting cell population, attempts may be made to stimulate the tumor cells to divide by administration of one drug or hormone in order to make them more vulnerable to treatment with a second drug while they are traversing either the $G_1$ or other phases of the cell cycle. There are various examples of efforts to recruit resting tumor cells into the proliferative pool in order to increase susceptibility to drug therapy (121-125).

Many other examples illustrate potential mechanisms for enhancing the action of one agent by another. Such mechanistic considerations are useful generators of therapeutic hypotheses. In the clinic, however, such hypotheses have generally been tested in a rather casual fashion. The methotrexate-5-FU experience is an instructive example. Interest in this combination intensified a few years ago with the demonstration that sequenced administration of the two agents led to enhanced cytotoxicity in vitro and therapeutic synergism in vivo (97,98). When the clinical trials began, however, the relevant associated laboratory correlative studies were not carried out and pertinent controls were absent from nearly all trials. The result several years later is persisting confusion concerning the real efficacy of sequenced MTX-5-FU and also concerning the relevance of the original biochemical hypotheses to the clinical observations (100).

About 10 years ago, certain studies alleged a kinetic advantage for the combination of vincristine and bleomycin; vincristine, it was held, could synchronize a tumor cell population, and bleomycin given 6 hours after vincristine would have enhanced selectivity against the synchronized cells, which by then should be in a more vulnerable phase of the cell cycle (126). This kinetic hypothesis

was never tested adequately in human tumors in vivo, nor was the two-drug combination ever subjected to controlled clinical testing to define the importance of sequencing. Instead it was simply adopted as the core of several multiagent regimens; eventually these regimens were consigned to oblivion after continued experience showed lack of significant efficacy (127,128).

The use of hormones to stimulate cell division and DNA synthesis in order to render the tumor more sensitive to cytotoxic drugs has yielded promising results in breast cancer (129,130,168). Until recently, however, controlled evidence for the possible superiority of sequenced hormonochemotherapy was entirely lacking (168); even now, the contribution of the sequencing itself (as opposed to the simple addition of the hormonal component to the chemotherapy) remains unresolved. In addition, the lack until recently of pertinent correlative laboratory studies to verify in vivo the hypotheses postulated for the hormonal-cytotoxic interaction has made the clinical results difficult to interpret and their relation to the hypothesis uncertain.

The observation that vincristine may promote the intracellular retention of methotrexate in vitro (131) and that the administration of these agents in combination provided schedule-dependent synergism against L1210 in vivo (114) spawned a whole generation of clinical protocols. The existence of the postulated interaction was never verified in vivo in patients, nor to our knowledge was an effort made to establish that vincristine administration enhanced the clinical effectiveness of methotrexate.

One could continue almost indefinitely with such examples. The point is that a postulated mechanism of interaction between two or more drugs—whether the interaction is on a biochemical, pharmacokinetic, or cytokinetic basis—may obviously be valid motivation for a clinical trial. In fact, these kinds of hypothesis-based trials may be our best hope for further improvements in combination chemotherapy using the available drugs. The hypothesis should, however, be studied as a serious component of the clinical trial, and the trial itself should be designed to yield a firm conclusion concerning the relative clinical effectiveness of the combination compared to the components alone or an otherwise suitable control group. Having such information, we are in a far better position to know where to go next. It matters both that a combination is or is not more effective than its components and also, whichever of these two results occurs, that laboratory evidence suggests or refutes the presence of the postulated mechanism. If the combination is a clinical advance and if the postulated mechanism is present, results are at least consistent with a cause-and-effect relationship; the evidence would be even stronger if a differential effect on tumor and normal tissue could be demonstrated, although admittedly this may be difficult in practice. If the combination is superior but there is no evidence for the mechanism, then there must be some other basis for the effect. These two outcomes might well suggest very different next-generation studies. If, on the other hand, the trial is clinically negative but there is evidence for the proposed interaction, the hypothesis is refuted. Lastly, if the combination is not superior and no evidence for the proposed mechanism exists, the hypothesis has received an inadequate test. Doing trials in this way wherever possible would be a very considerable departure from past practice, but would leave us very much richer for the effort.

## Strategies to Circumvent Drug Resistance

The characteristics of tumor cells that render them insensitive to the killing effect of anticancer drugs at levels achievable in vivo subsumes nearly the whole problem of chemotherapy; only the access of active drug to the tumor cells themselves lies outside this general area. A large body of experimental evidence suggests that the emergence of drug-resistant phenotypes in tumors initially responsive to chemotherapy is a major cause of therapeutic failure (132). Although comparable information from the clinic is less comprehensively and rigorously analyzed, common clinical experience, as well as data obtained from cell lines from patients clinically resistant to chemotherapy (133), is consistent with the importance of acquired drug resistance as a major limiting feature of chemotherapy's curative potentials in initially sensitive tumors.

A modest effort has been directed toward characterization of the kinetics of appearance of drug resistance with combinations in comparison to single agents. One example is provided by the combination of methotrexate plus chloroTIC, in which leukemic mice were treated over a series of transplant generations with the drugs individually or in combination. As measured by survival-time response, resistance occurred more rapidly with the individual drugs than with the combination of drugs (26).

In another study, a comparison was made of the ability of cytarabine and cyclophosphamide to delay the origin of leukemia (L1210) cell resistance (46,134). Treatment of the leukemic mice with cytarabine for 5 consecutive days, followed by transplantation of the tumor on the tenth day and repetition of the treatment with cytarabine in subsequent transplant generations, resulted in complete resistance to cytarabine by the fourth generation. In similar manner, on daily treatment with cyclophosphamide over successive transplant generations, complete resistance to cyclophosphamide occurred by the fifth generation. On treatment with the combination of cytarabine plus cyclophosphamide, the appearance of resistance to cytarabine was delayed for at least two transplant generations and the origin of resistance to cyclophosphamide was delayed for at least one generation.

Skipper et al (132) and Schabel et al (135) considered possible strategies for the eradication of a maximum number of drug-resistant tumor cells with a limited number of effective, although noncurative, drugs. Initial ther-

apy is followed by a change to other drugs that are cytostatic to both residual tumor cells and mutant tumor cells that have become resistant during the first course of treatment. For example, treatment of iv inoculated leukemia L1210 with cytarabine plus 6-thioguanine yielded therapeutic synergism, but the treatment ultimately failed when tumor cells resistant to both drugs became predominant. However, with the administration of two courses of cytarabine plus 6-thioguanine, followed by cyclophosphamide plus carmustine, there were marked increases in life span and a high percentage of cures (132). Similar results were obtained with sequential treatment involving leukemia P388 (135).

In any alternated scheme, the question of timing (when to alternate?) obviously arises. In the absence of acquired resistance, the net amount of cytoreduction to be expected from a treatment can be related quantitatively to the cell kill per course (a direct function of dose), the number of courses, the doubling time of the residual cells, and the interval between doses (136). In one approach, application of this relationship to advanced L1210 leukemia led to institution of the second regimen at the nadir of cell kill from the first regimen; this proved to be the most efficacious way of preventing the emergence of resistant cells. In general, computer modeling and experimental results have suggested that using the highest tolerable doses to achieve the greatest possible cell kill, and cycling alternating regimens as quickly as possible to avoid substantial degrees of regrowth of surviving cells, will result in the most predictable eradication of sensitive and partially resistant cell populations.

The use of sequential therapies to suppress the emergence of resistance has recently been placed on a firmer theoretical foundation by the mathematical modeling of Goldie and Coldman (137-140), which addresses explicitly the problem of tumor cell resistance. As applied to the killing of cancer cells by cytotoxic drugs, the somatic mutation theory predicts that maximally effective therapy should result from the use of as many active agents as early in therapy as possible. The theory itself does not prescribe details concerning the construction of specific combinations, and many strategies are possible, depending upon the specific characteristics of the individual agents. Since many cytotoxic drugs have overlapping toxic effects, however, the construction of two (or more) separate two- to four-drug combinations, delivered in alternating fashion, has been the subject of particular attention (138). It turns out that the mathematical model imposes a rather strict requirement on the therapeutic characteristics of alternated combinations; in order for the alternated strategy to exhibit superiority, the combinations to be alternated must be approximately equivalent in therapeutic efficacy. In addition, although the requirement that the combinations be non-cross-resistant is not an absolute one, it is clear that the more non-cross-resistant, the better. In the limit where two combinations of equal therapeutic efficacy show complete cross-resistance, an alternated strategy would obviously be no better than the use of only one of the combinations. In general, the predictions of the somatic mutation theory are in good agreement with experimental data from the therapy of transplantable tumors (141).

The possible advantages of alternating therapy with non-cross-resistant regimens have long intrigued clinicians, and attempts to test this strategy began well before the initial publications of Goldie and Coldman. As noted elsewhere, the design of nearly all such schemes has been on an intuitive basis, with little consideration of some of the most important requirements of the formal theory, which have only emerged fairly recently. Unfortunately, the interval between alternation of combinations has in general not been short, the regimens to be alternated have rarely been shown to be in fact non-cross-resistant, and the therapeutic equivalence of the alternated regimens has rarely been established. In addition, the strategy has been attempted inappropriately in diseases where the very high prevalence of de novo resistant cells gives no reason to believe that alternation will confer any advantage at all. In light of these considerations, the apparent success of alternating combinations in a few instances may seem like exceptional strokes of luck, rather than perhaps the isolated circumstances where the actual conditions perhaps corresponded most closely to the requirements of the hypothesis.

The recent interest in drug resistance and issues of dose intensity has led to a more detailed reconsideration of causes of therapeutic failure to drug combinations. Skipper has shown clearly in mouse mammary tumor models treated with the combination of cyclophosphamide, doxorubicin, and 5-FU that treatment failure is not accompanied by uniform expression of drug resistance to all three components of the combination; in general, tumor which regrows in the face of continued combination treatment is resistant to doxorubicin but sensitive to cyclophosphamide or 5-FU (142,143). The implication of such findings is that adjustment of the dose intensities of the individual drugs might further improve results, since clearly the dose intensity of the regimen as given is not sufficient to extinguish some of the singly resistant populations.

Clinically, there is very little information of this kind, because oncologists generally do not treat patients failing a combination of drugs with one element of that combination. Certain experience with salvage therapy, however, has made it abundantly clear that failure to kill only the drug-resistant subpopulation is not the entire problem. Patients with Hodgkin's disease initially responding to treatment with MOPP (mechlorethamine, vincristine, procarbazine, and prednisone) and then relapsing more than a year following induction are generally re-treated with the same drug regimen with good expectation of success (144). Patients who receive CMF (cyclophosphamide, methotrexate, and 5-FU) as adjuvant therapy for breast cancer and then relapse > 12 months later and are

re-treated with CMF have response rates not very different from patients never previously exposed to cytotoxic agents (145). These data indicate that under some circumstances a substantial fraction of the surviving cell population may still be drug-sensitive; in other words, the combination was not given with sufficient intensity to eradicate the sensitive cell population.

One of the most important controllable variables relating to drug resistance is dose intensity. In the laboratory, the importance of dose has been a closed issue for years: the relation of dose intensity to effectiveness of treatment is essentially invariable (33). Many clinicians have been slow to put these lessons into practice, no doubt because of fear of undesirable levels of toxicity. Much evidence, however, points to the clinical importance of dose intensity. The recent analysis of Hryniuk and Bush (146) supports a quantitatively significant effect of dose intensity on results in CMF-containing regimens in metastatic breast cancer; although this analysis is based on retrospectively collected trials data, the striking quantitative relation between dose intensity and effect is quite compelling. Data from the chemotherapy of Hodgkin's disease suggest similar conclusions (147). Escalation of the cisplatin dose appears to carry with it a corresponding increase in response rates in testicular cancer (156); regimens incorporating very high-dose cisplatin have unprecedented levels of activity in poor-prognosis germ cell tumors (148,149). In refractory tumors such as colorectal carcinoma and melanoma, a clearcut increase in response rate accompanies escalation of alkylating agent doses to levels requiring bone marrow rescue (150,151).

## CONCLUSIONS

In the clinical development of combination chemotherapy, transplantable tumor models have been an invaluable tool. With them, experimental chemotherapists have established many basic principles in treatment which seem to have applicability to clinical cancer. In the models, the superiority of well-chosen drug combinations over single agents; the importance of dose intensity, ratio, and scheduling; and the effect of body burden on treatment effectiveness are consistent findings for which there are in many cases corresponding clinical results. For chemotherapeutic purposes, most of the preclinical tumors are probably better models of the relatively high growth-fraction hematologic malignancies, germ cell tumors, and certain other aggressive cancers than they are of the more slowly growing, relatively low growth-fraction epithelial solid tumors. Although the models do exhibit considerable heterogeneity in various phenotypic characteristics, including sensitivity to chemotherapy, even the relatively resistant model tumors seem much more sensitive to existing drugs than most human cancers. A clinician reading an experimentalist's description of a resistant transplantable tumor that has a very low response rate to drugs cannot help being struck by the fact that even here drug treatment may increase survival compared with control (33). Because of these differences, extreme care in interpretation must accompany all attempts to apply the laboratory experience with specific combinations to the clinic.

It is clear, however, that many of the general lessons learned in the models have been applied in the clinic too casually or not at all. Admittedly the clinic is a very difficult laboratory, and we do not mean to minimize the real problems involved. We do think, however, that the following issues need further attention.

(*a*) It seems likely that further progress can be made against tumors in Group A, and perhaps the more sensitive ones in Group B, by more effective use of currently available drugs. Recent regimens combining more aggressive drug doses with attempts to give as many active agents as possible during the initial stages of treatment have produced suggestively better results in the aggressive and intermediate non-Hodgkin's lymphomas (152,153), Hodgkin's disease (154), and the germ cell tumors (155) than have been achieved by conventional programs. A recent report from the Vancouver group (153) shows that alternating combinations are not the only way to accomplish this, even when overlapping toxicity is substantial. In a very preliminary report (156), the use of multiple alkylating agents in a high dose with bone marrow rescue looks extremely active against metastatic breast cancer. The delivery of certain drugs by the regional route in the appropriate clinical circumstances promises to be an effective means of selectively increasing exposure of tumor cells to cytotoxic drugs.

In appropriately selected tumors, the lessons of the Goldie-Coldman model should be incorporated further into protocol planning. Formal controlled tests of the hypothesis are likely to be quite difficult to construct; the requirement of therapeutic equivalence, in particular, is difficult to satisfy. Nevertheless, the major predictions of the somatic mutation theory may be used to aid the empirical design of combinations. It seems most likely that tumors will respond to this approach more or less in proportion to their general sensitivity and to the number of truly non-cross-resistant agents that can be incorporated at effective doses into combinations.

(*b*) For many of the tumors in Group B, prospects for the purely empirical development of curative combinations for disseminated disease using existing drugs do not appear very bright. Available agents do not appear to have the requisite activity (at least not at conventional doses) to permit the empirical construction of combinations that will induce significant complete response rates. Even when total response rates are quite respectable, as in head and neck carcinoma, the relatively short duration of remission signifies a depressing ability of the tumor to evolve drug-resistant subpopulations. Sufficient numbers of active and non-cross-resistant agents do not exist for many of these tumors to permit an aggressive

approach with alternating combinations similar to that which has proved successful or promising in other tumor types. And even if such combinations were available, the advanced age and frequent presence of significant medical problems in many patients with these diseases might be an obstacle to their use.

Because of the potential of multimodality therapy in Group B, however, efforts to develop more effective combinations should clearly continue, even if the likelihood of developing curative combinations for disseminated disease is low. And, as previously outlined, there are reasons for optimism. Evidence for increased activity of drugs at very high doses and the possibility that even more active combinations of high-dose components may be constructed at this extreme end of the dose spectrum suggest that meaningful increases in complete response rates may not be out of the question. In a similar vein, the rigorous development of combinations based on specific hypotheses concerning mechanisms is a difficult but potentially promising area. Combinations of drugs with hormones and drugs with immunomodulators may benefit particularly from this approach. As noted previously, however, when such trials are mounted, it is of the utmost importance to verify both that the combination is or is not superior to its constituents alone, and, simultaneously, that evidence for the postulated mechanism is or is not present.

(*c*) Efforts at combining minimally active agents for the tumors in Group C have been generally unproductive. Despite years of exploratory efforts in these tumors, demonstrations of possible effectiveness have thus far been limited to certain combined modality settings (162,163), and even here it is not clear that the effect is of significant magnitude (164) or that the combination as such contributes materially to the results (165). Obviously, research programs should emphasize the discovery and development of effective new agents for these diseases. Again here, hypothesis-based trials with existing agents are also a rational way to proceed, provided that the hypotheses are solidly grounded and studied in parallel with the clinical trial.

(*d*) The cross-resistance patterns of drugs contemplated for use in combination should be better defined. Little will be achieved from using cross-resistant agents together. In this regard, we need much more information about actual patterns of cross-resistance in human tumors. Data derived from nonhuman cell lines are potentially helpful because they illustrate many possibilities that one might observe in the clinic (166). Such data cannot, however, replace direct information from clinical material. Multiple mechanisms of resistance to many drugs certainly exist and whether two drugs will exhibit cross-resistance or not may well depend upon the mechanism. Resistance arising clinically may have quite different frequency distributions of the possible mechanisms than resistance resulting in cell lines exposed to laboratory selection procedures. It goes without saying, of course, that effective techniques for reversing resistance would be a major step forward.

(*e*) We badly need to develop a general theory of combination chemotherapy that will be usable in the clinic. Although the problem may be posed very simply, it is one of formidable complexity: if we have N active agents $D_1$, $D_2$, . . . $D_N$ for a given cancer, how may they be combined to maximize survival? It would seem that with certain assumptions about (or knowledge of) the interactions among the agents, this problem should be approachable, provided we have reliable information about the therapeutic characteristics of the individual agents in the disease. As things stand now, however, we do not have adequate methods of deciding even how many of the available agents should be included. This is clearly a key point, since the inclusion of a relatively less active agent may be desirable if it is non-cross-resistant with the more active agent(s), but undesirable if its inclusion limits the deliverable dose of the more active drugs. A closely related and equally crucial aspect of the problem is determination of the optimal dose ratio of the drugs that are included in the combination. For combinations whose components have at least one overlapping toxicity that becomes dose-limiting when the drugs are used together (eg, the myelosuppression seen with cyclophosphamide and doxorubicin in breast cancer, cisplatin and etoposide in SCLC, and cisplatin and cyclophosphamide in ovarian cancer), there is simply no available algorithm for deciding what the dose ratio of the two drugs in combination ought to be. For combinations of three or more agents, the problem is correspondingly more complex. Dose ratio is of much more than merely academic interest; the steep dose-response curves of most anticancer agents and the demonstration in transplantable models that dose ratio may be an important determinant of effectiveness make this issue of significant practical importance. In the actual construction of combinations, many investigators appear to proceed by giving greater weight to the more active components. While not unreasonable, this procedure lacks any quantitative basis. The problem is most significant when overlapping toxic effects impose a constraint on the total dose of both drugs that can be given together. With certain combinations, where toxic effects do not overlap at all, each agent in the combination can (and should) be given as close to its full single-agent dose as possible.

As noted before, Skipper has described persisting drug sensitivity in model tumors failing combination chemotherapy to individual elements of that combination. Skipper (167) and Goldie (personal communication) have both emphasized the desirability of obtaining comparable information in the clinic. In the absence of reliable in vitro techniques, doing so would require re-treating patients failing combinations with individual elements of the combination at full dose. The demonstration of persisting sensitivity to any of the single agents would most likely indicate that dose ratios in the original combination were

suboptimal. Whether this kind of information would permit the construction of more effective combinations, however, is open to question; the extensive heterogeneity of human tumors might give many different answers. For example, in a group of patients with breast cancer responding to and then failing treatment with CAF (cyclophosphamide, doxorubicin, and 5-FU), it might well happen that a number of different patterns of persisting sensitivities to cyclophosphamide, doxorubicin, and 5-FU, singly and in pairs, might emerge. If this should occur, the implication would be that general rules may not be possible and, in theory at least, dose ratios would have to be tailored to the individual patient. The impracticality of this, at least with current technology, needs no elaboration. As techniques for the noninvasive assessment of the metabolic effects of drugs become more sophisticated, however, adjusting relative doses in this way may not be out of the question for the oncologist of the future.

In the absence of a comprehensive theory of combination chemotherapy, response surface methodology (RSM) may offer an approach to optimizing dose ratios and scheduling. Elsewhere in this symposium, Carter discusses the possible applications of RSM to therapeutic research in cancer. In principle, this approach is applicable to clinical trials, and the Mid-Atlantic Oncology Program has an ambitious study under way which seeks to define an optimal CMF regimen for metastatic breast cancer. Requirements in terms of patient numbers, while substantial, are very much less than they would be for conventional randomized trials asking the same sets of questions. Whether RSM will prove feasible for clinical trials is an important unresolved issue.

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# Application of Theoretical Models to Chemotherapy Protocol Design

J. H. Goldie[1,2,*] and A. J. Coldman[1,3]

**The somatic mutation model of cancer chemotherapy predicts that under certain conditions some sequences of chemotherapy drug administration will be superior to others. Alternation between two non-cross-resistant combinations that are equivalent in effectiveness is one such sequence. This method was utilized in a number of clinical trials based primarily on intuitive perceptions on how treatment failure due to drug resistance can be minimized. A survey of a number of recently published trials employing some form of alternating chemotherapy indicates that a majority of them suggested an advantage to this approach compared to sequential therapy, although in most studies this benefit was small. Most of the studies, whether or not supportive of the concept of alternating therapy, could not be considered a fair test of the theory. Reference to theoretical models of chemotherapeutic action may provide assistance in devising clinical trials that will rigorously test the concept of alternating chemotherapy to see to what extent this technique will improve treatment results in selected neoplasms. [Cancer Treat Rep 70:127–131, 1986]**

The purpose of mathematical models in science is to provide a deeper insight into natural phenomena and through this insight make accurate predictions about the behavior of such phenomena. Verification of these predictions will produce further understanding of the processes being studied, which in turn may lead to modification and enhanced sophistication of the basic model. In fact, a valid mathematical model must yield a set of hypotheses (or predictions) which can be subjected to unambiguous disproof under the appropriate experimental conditions. The experiment has to be a fair test of the hypothesis, however, which requires careful attention to the assumptions of the model when the experiment is planned. It should be remembered that anyone carrying out as complex an experiment as a clinical trial of cancer chemotherapy must be working on the basis of some kind of model of the disease and treatment effects. It seems to us that it is clearly better to utilize models in which the assumptions and relationships are explicit and quantifiable rather than ones that are ill defined and purely phenomenological.

In previous publications we have outlined a model for cancer chemotherapy effects which, among other assumptions, postulated that a major factor in treatment outcome was the presence or absence of drug-resistant mutant cells (1,2). The origin of these cells was assumed to be a result of spontaneous mutations in accordance with the principles described by Luria and Delbruck (3).

Supporting evidence for this assumption is available from a large amount of data from experimental chemotherapy trials (4–6). Much of this information was developed by Dr. F. M. Schabel, Jr, and his colleagues, utilizing a variety of transplanted animal tumors and a large number of different antineoplastic agents. The information from experimental chemotherapy seems unequivocal, but it is conceded that much less data are available from clinical experiments. Nonetheless, a growing amount of evidence from studies in man is compatible with the hypothesis that treatment failure in clinical situations may have many similarities to the experimental one (7–9). It would seem reasonable, therefore, to develop theoretical approaches to optimizing treatment strategy and then examine to what extent these techniques will yield superior treatment results.

## Predictions of the Somatic Mutation Model

The basic assumptions and the underlying theory of the model have been described in detail elsewhere (1,2, 10,11). We will discuss here some of the principal predictions regarding chemotherapy strategy that can be inferred from the model with emphasis on one particular stra-

[1]Divisions of Medical Oncology (*J. H. Goldie*) and Epidemiology and Biometry (*A. J. Coldman*), Cancer Control Agency of British Columbia, Vancouver, Canada.

[2]Department of Medicine, University of British Columbia, Canada.

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[*]*Reprint requests to:* Dr. J. H. Goldie, Cancer Control Agency of British Columbia, 600 W 10th Ave, Vancouver, British Columbia, Canada V5Z 4E6.

tegic set, the role of specific sequencing of drug combinations in minimizing treatment failure due to drug resistance.

#### *Relationship Between Tumor Burden and Curability*

As a tumor population increases over time there will be an increasing likelihood that at least one drug-resistant clone will spontaneously appear within the neoplasm. With further increases in tumor size more clones will emerge, and some of them will mutate to higher levels of resistance. Elapsed time and increasing tumor mass will mitigate against successful chemotherapeutic effect. Other phenomena than drug resistance will be aggravated by increasing tumor mass, but the somatic mutation theory predicts a specific quantitative relationship between tumor size and probability of cure. This relationship has been found to be present in a number of experimental tumors undergoing treatment with a variety of antineoplastic drugs (12). Whether the same close relationship between tumor mass and curability exists in clinical cancer chemotherapy is not known, but there is certainly a strong inverse correlation between tumor burden and curability in a number of human malignancies (13).

#### *Enhanced Curative Potential of Combination Chemotherapy*

The same mathematical function that defines curability with respect to tumor size can also be used to develop a rationale for combination chemotherapy. Probability of cure is related to both tumor size and the mutation rate to resistance. The lower this latter quantity is, the greater the probability of cure for any given tumor size. The use of multiple agents with independent loci of action (and different pathways for resistance) will have the effect of lowering the net mutation rate to resistance by the neoplastic cells. The model predicts that the major impact of combination chemotherapy will be to increase cure rates (where this is a realistic option) and not just to increase response or remission rates. The increased effectiveness for combination chemotherapy in both clinical and experimental trials has been abundantly documented, and cannot be considered a matter for serious dispute at this time (14).

#### *Enhanced Therapeutic Potential of Fixed Alternation Schedules Using Equivalent Non-cross-resistant Drug Combinations*

When one examines the mathematical theory underlying combination chemotherapy effect, it is possible to develop a rationale for certain sequences of drug administration, provided a number of fairly stringent criteria are met. This is one of the more controversial predictions of the somatic mutation model, and, therefore, the assumptions involved and the extent to which these have been tested in clinical trials will be examined in more detail. Parenthetically, we should add that alternating chemotherapy trials of various types were undertaken well before the publication of the mathematical theory. It appears it is the attempt to develop a rationale for this approach that has provided controversy, as opposed to the conduct of the clinical trials themselves.

If one has two drugs (or drug combinations) designated A and B and if they cannot be administered concurrently (because of toxicity), then on average the most effective way to utilize the agents will be to sequence them in an alternating fashion ABAB . . . (or BABA . . . ) as opposed to any other fixed strategy (2,10). For this approach to be valid, the drugs must be quantitatively equivalent in their effect, ie, produce the same log-kill on susceptible cells, and have the same mutation rates to resistance. In addition, the sensitive tumor cells and the resistant subsets must be kinetically similar to each other. Maximum benefit will be seen when A and B are totally non-cross-resistant, but there may still be benefit even if considerable cross-resistance is present (15). The basis for the increased effectiveness of the alternating strategy is that this is the technique that will allow the least amount of regrowth of the drug-resistant subclones in between applications of treatment. This minimizes the risk that one resistant subclone will be permitted a prolonged period of unimpeded growth and hence have an increased opportunity to mutate to a higher level of resistance.

Small deviations from absolute symmetry will not affect the superiority of the alternating strategy. As this symmetry is increasingly violated, a point will be reached when alternating chemotherapy no longer emerges as the optimal strategy in an individual case. The effect of asymmetry in this connection has been analyzed by Day (16), who has indicated some of the circumstances when alternating chemotherapy may be bested by some other strategy, ie, AABAAB, etc. The practical problem in utilizing an unbalanced strategy, however, is that it requires a great deal of specific information about the nature of the tumor system that is being treated. By and large, this information is not going to be available to the clinical therapist.

We can say that alternating non-cross-resistant chemotherapy does appear to have a rational basis, but for it to work in practice a number of relatively stringent conditions have to be met. These conditions are not impossible, but neither are they likely to be achieved by the ad hoc assemblage of multiple-drug protocols which are simply assumed to be non-cross-resistant and quantitatively equivalent. Where the quantitative criteria for alternating chemotherapy can be satisfied, then it has been possible to demonstrate the effectiveness of alternating chemotherapy strategies in experimental tumors (16). In the next section, we will examine to what extent clinical experiments support the utility of this method or otherwise.

## Applications of the Concept of Alternating Chemotherapy in Clinical Trials

In recent years, a number of clinical trials have been published in which some type of "alternating" chemotherapy was employed (17–35). All of these studies were initiated prior to the publication of the theoretical analysis of alternating chemotherapy discussed above. It should not be surprising, therefore, that many of these studies do not really provide a rigorous test of the underlying concept. Some of the published studies did not employ a "fixed" schedule, but used a variable number of initial treatments to maximum therapeutic effect before switching to the alternate treatment arm (24,35). In a number of studies, non-cross-resistance between arms was assumed primarily on the basis of the apparent differences in the classes of the constituent drugs. Quantitative equivalence of therapeutic effect was usually not regarded as a major requirement and was inferred on the basis of historical results (frequently as assessed by results published in the literature as opposed to institutional historical data).

Some trials combined hormones, nonspecific immunotherapy or radiotherapy as part of one or both arms, making the impact of the chemotherapy component difficult to evaluate (20,30,32,34). In other trials, alternating chemotherapy was introduced only as a so-called "maintenance" arm after initial remission induction (23). In at least some studies, the term "alternating" is open to some semantic interpretation because the phrase alternating chemotherapy can be defined by subjective distinctions employed by the investigator. That is, is alternating high-dose methotrexate with bleomycin, doxorubicin, vincristine, cyclophosphamide, and dexamethasone (33) really an "alternating" regimen or might it be more useful (and accurate) to simply describe it as an intensive multiagent protocol given at 3 weekly intervals? Most of the cited studies are basically reports of complex multiagent protocols which employ a considerable variety of agents at a substantial dose intensity. That many of them appear to be yielding superior results to older, less intensive programs is not surprising, and would also be predicted by the somatic mutation theory. However, we wish to look specifically at this question: does strict one-to-one alternation of two equivalent drug arms yield better results than would be obtained from the sequential use of these protocols?

To answer this question systematically, we first require that the disease to be treated be susceptible to significant therapeutic amelioration by a number of drugs or drug combinations. If the tumor type is very refractory to all of the known drugs and drug combinations (ie, advanced pancreatic carcinoma), then it is highly unlikely that sequencing techniques are going to generate discernible gains. Ideally, one would wish to see diseases for which combinations exist which have some curative potential or at least are capable of producing long-lasting remissions in a significant proportion of patients. This, of course, reduces the number of types of neoplasm that will be suitable for testing.

The treatment arms that are to be designated as the two components should have been compared initially for effect in adequate numbers of previously untreated patients. There should be comparable remission rates, duration of remission, overall survival, and, where feasible, cure rates. The degree of cross-resistance can be assessed by using each of the arms as the salvage protocol for the other (from the basic considerations of the somatic mutation model it can be easily shown that such use will, if anything, tend to underestimate the degree of non-cross-resistance). Also, within the context of a prospectively randomized trial, two different sequences should be compared, ie, ABABAB . . . with AAAAABBBBB. Some arbitrary decisions about starting sequences will have to be made in order to forego the necessity of comparing BABABA . . . with BBBBBAAAAA, etc. Finally, the symmetry between the two treatment arms must also be related to time so that the intervals between ABA, etc, are the same as between AAA. This implies comparable toxicity between the protocols, which will in turn ensure equivalent drug dose intensity over the period of treatment.

Given the formidable requirements to demonstrate the utility of alternating sequence protocols, it is not surprising that most studies cannot be considered to be tests of the hypothesis. It is perhaps more surprising that at least a few studies meet a number of the conditions. These include a study of advanced breast cancer (28), small cell carcinoma of the lung (25), and Hodgkin's disease (29). All of these trials yielded results that suggested superior therapeutic effect for the alternating protocols, although in the lung and breast studies the benefits were small. Results in Hodgkin's disease were dramatic, however, and this particular study was the one which has come closest to meeting the theoretical study requirements. That is, the investigators first established the near equivalence of effect of mechlorethamine, vincristine, procarbazine, and prednisone (MOPP) versus doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD), by using both combinations against previously untreated patients and assessing response rates and survival with each protocol. The alternating protocol was then compared against MOPP therapy alone in a prospectively randomized trial. The comparison here, therefore, was ABABAB . . . against AAAAAA . . . . The model predicts for the superiority of alternation over AAA . . ., but also predicts for the superiority of alternation over AAAAAABBB . . . , which, however, was not evaluated in the study. This strategy (AAAAAABBB . . . ) would be expected to be better than the strict A strategy, but less effective than the one-to-one alternation.

Of the total number of alternating studies reviewed here, ten yielded results that were considered positive for the alternating sequence (18,25–31,33–35) and six pro-

duced results that showed no improvement (19-24). In two studies, it was not possible to draw any inference one way or the other (17,32). However, in no reported study was the alternating program found to be inferior. As mentioned previously, most of the studies showing increased benefit are probably due to the effect of an intense and complex "treatment field" rather than as a result of sequencing. This, however, does not minimize their importance and usefulness.

A more refined test of the alternating theory would be to take a demonstrably effective protocol (ie, MOPP/ABVD) and by applying basic principles modify the protocol in the direction of producing better therapeutic results. In MOPP/ABVD, a month elapses after the initial treatment is given before the non-cross-resistant agents are introduced. The mathematical prediction is that utilizing the doxorubicin-containing component earlier, that is, on Day 8 or 14, should increase the effectiveness of the whole protocol. By how much and whether this would be detectable in a clinical trial is not clear. Some early data based on the MOPP/ABV Hybrid protocol (doxorubicin on Day 8) (36) and "half-MOPP half-ABVD" (Day 14) (37) suggest trends in favor of better results for the earlier alternating protocols. A study sponsored by the National Cancer Institute of Canada is being mounted in a number of Canadian centers to test this concept directly. Hybrid will be compared in a prospective randomized trial with MOPP/ABVD. As results with MOPP/ABVD are already very good, demonstrating enhanced benefit with a similar protocol may be difficult, but a statistically significant improvement in favor of Hybrid would be useful supporting evidence for the approach and for the underlying theory.

## CONCLUSION

We would not wish to minimize the difficulties in taking theoretical models and applying them in a useful fashion to real clinical situations. We would suggest that a subtle and complex treatment strategy such as alternating chemotherapy might not be given an adequate evaluation without the guidance of appropriate mathematical models. The model helps define the essential ingredients of such a strategy and how it may be both tested and refined. Empirical methods will be less likely to achieve this and will require a much greater component of time-consuming trial and error. Moreover, it would be unfortunate if a potentially useful chemotherapeutic approach was discarded prematurely because it had not been properly evaluated.

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# Review of the Application of Response Surface Methodology in the Combination Therapy of Cancer

**Walter H. Carter, Jr* and Galen L. Wampler[1]**

The value of a dose-response curve in understanding the effects of a single drug is clear. Typically for a cytotoxic agent, survival time or probability of a favorable outcome increases as the dose is increased until a maximum response is reached. Then, due to toxicity of the drug, as the dose is increased from this point the survival time or the probability of a favorable outcome decreases. Thus, when treatment consisting of a single drug is associated with lethal toxicity, it is clear that a decrease in the dosage level should be associated with a decline in the incidence of toxicity and a move toward the optimum dose. This seems to be the justification for treating with the maximum tolerated dosage. It is important to emphasize that the justification for this comes from our understanding of the dose-response curve. Unfortunately, properties of the dose-response curve for a single agent are not necessarily associated with the dose-response surface for a combination of agents. The application of the collection of statistical procedures known as response surface methodology (RSM) to describe the dose-response relationship for a combination of agents has the potential of providing valuable insight into how agents can be combined more effectively. It is the purpose of this article to review the use of RSM in oncology. The format of this review will be as follows:

1. Definition of RSM
2. Historical review
3. Definitions relating to drug actions and interactions
4. Adaptation of RSM to cancer chemotherapy
5. Applications
6. Summary

## Definition of RSM

RSM is a collection of mathematical and statistical methods which has been developed and used historically to aid in the solution of particular types of problems pertinent to engineering and scientific processes. The methods include experimental design, statistical inference, and mathematical optimization techniques, which when combined enable the experimenter to make an efficient empirical exploration of the process on which interest is centered. This approach to defining relationships between variables has been mainly used in industrial settings. Problems faced by investigators in the chemical industry led to the modern development of these methods. In order to maximize yields of chemical products, the proper levels of input variables such as temperature, pressure, heating time, etc., are required. When three or more variables require simultaneous adjustment for optimization, these methods are efficient. Hence, the applications to combination chemotherapy seem obvious.

The essence of the response surface approach is that a mathematical relationship (model) exists between the levels of treatment and the outcome observed. Thus, one generally works with causal models where the levels of treatment or design variables are controlled by the researcher. In RSM the treatment variables appear in a regression sense as opposed to an analysis of variance sense. By this it is meant that the model is parameterized in such a way that the actual levels of treatment are related to the treatment outcome so that the effects of different levels of treatment can be predicted. This is as opposed to a parameterization whereby only comparisons between different treatment groups are permitted. The importance of this distinction lies in the fact that once the levels of treatment are related to the treatment outcome in a regression sense, RSM can be used to explore the resulting response surfaces and obtain information not forthcoming from other analyses.

There are several stages to the usual response surface analysis:

(*a*) Experimental design
(*b*) Model selection and parameter estimation
(*c*) Model verification
(*d*) Exploration of the fitted response surface

These phases of a response surface analysis are given in the chronological order performed, once it has been determined to employ the RSM experimental approach. From the point of view of statistical development, it is more natural to consider the estimation of the model parameters prior to the development of the experimental

[1]Departments of Biostatistics, Medicine, and Pharmacology and Toxicology (*W. H. Carter, Jr*) and Medicine (*G. L. Wampler*), Medical College of Virginia, Virginia Commonwealth University, Richmond.

**Reprint requests to:* Dr. Walter H. Carter, Jr, Box 32, MCV Station, Richmond, VA 23298.

design. This follows since once the estimation procedure has been established and estimators determined, the statistical properties of the estimators as a function of the location of the design points can be ascertained. As a result, it is often possible to determine the placement of experimental points in such a manner as to optimize certain desirable properties of the estimators.

## Historical Review

In a discussion of a paper by Mantel (1), Box (2) in 1958 suggested the potential usefulness of regression analysis in gaining an understanding of the relationship between levels of treatment and their effect. With an appropriate model it would be possible to gain an indication of the effect of the drugs in different ratios and doses than those used in the experiment described by Mantel. Box further suggested that a more realistic analysis might include modeling the relationship among treatment levels and various manifestations of toxicity in addition to the endpoint of major interest, eg, survival. Once these models had been developed and their predictive ability verified, it would then be possible to determine treatment levels associated with maximum survival, subject to constraints placed on the incidence or levels of the various measures of toxicity.

These suggestions were not acted on for 8 years when Addelman et al (3) devised an experimental scheme for estimating the optimal combination of two drugs. Historically, theirs is the initial paper reporting on the use of such methods in this area of cancer research. Unfortunately, the authors illustrated their procedures with hypothetical data. Thus, they could report neither how well their model related treatment levels to survival times nor the accuracy of the estimates of optimal dosage.

Other writers have commented on the potential usefulness of these methods (4) or have noted the lack of application by biomedical researchers (5).

Carter et al (6) proposed the use of logistic regression and demonstrated a good fit to animal data. Later, Cox's proportional hazards model (7), which permits the more complete use of survival data rather than the somewhat arbitrarily defined acceptable response or lack thereof with the logistic model, was utilized (8). Development of the statistical methods for calculating the confidence region about the optimal treatment (9) and the confidence interval about the response at the optimal treatment (10) has further enhanced the utility of methods available for exploring response surfaces and statistically defining their properties.

Reports to date have demonstrated the adaptability of these methods to various types of animal combination chemotherapy studies (8,9,11-14) or combinations of radiation therapy and chemotherapy (15-17), and at the time of this report clinical studies using these approaches are under way in the Mid-Atlantic Oncology Program.

## Definitions Relating to Drug Actions and Interactions Based on Optimum Treatment Levels

### *Optimum Dose*

Through use of RSM it is possible to estimate the level of each component in a combination associated with optimal response, eg, probability of a favorable outcome or maximum survival time. It has become apparent that the optimum dose can change depending on a number of factors. With one drug the optimum dose can change only in a linear manner, ie, increase or decrease. With two or more drugs this change may take any direction within the treatment space. The ability to study these changes depends on the conditions defining an optimum response; this had not been accomplished previously due to inability of the methods used to systematically explore and statistically analyze the underlying dose-response relationship. For example:

1. If one is interested in long-term versus short-term benefit, one would assume that the optimum dose would be higher, but in the case of razoxane and hexamethylmelamine combination treatment of L1210 leukemia, the dose of the active drug (razoxane) is lower and the inactive drug (hexamethylmelamine) is higher as one optimizes for longer-term survival (18).

2. The optimum dose for treatment of early disease (which could be equated with treatment in the adjuvant setting) may be different from that required for treatment of advanced tumor. In studying the combination of 5-FU and cyclophosphamide (CTX), it was found that in L1210 and P388 leukemias a therapeutic synergism could only be demonstrated if there was a time interval between the administration of 5-FU and CTX. Experiments run in L1210 leukemia using a time interval of 24 hours with 5-FU given first showed the optimum dose to be CTX alone with first treatment of tumor on Days 2-4 (no therapeutic synergism). With later first tumor treatment, the optimum dose shifted to include 5-FU in combination with CTX. Conclusions from these experiments are the expected finding that treatment is less and less effective as the tumor becomes more advanced and the unexpected finding that two competing mechanisms appear to be involved. In the early tumor, a mechanism not requiring the participation of 5-FU predominates so that CTX alone is optimal treatment. Later, a mechanism involving the participation of 5-FU becomes the major contributing factor to longevity, resulting in the "jumping" of the optimum dose from one region of the treatment space to another (13).

3. There are different optimum doses with different schedules. This is a bit different from the situations discussed above, since schedule is always in the hands of the investigator. Consequently, the optimum treatment includes a specific schedule. Nevertheless, it is important to recognize that the direction the optimum dose takes in the treatment space as the interval between drugs is in-

creased or the time between treatment cycles is increased is a property of drug interaction. Heretofore this knowledge has been unavailable for its potential applications in suggesting mechanisms of drug interaction or for exploitation in the design of clinical regimens. An example of how optimum treatments change as the interval of time between two drugs is increased is provided by the study of the combination of methotrexate and teniposide utilizing an interval between drug administration. In this example, a triple variable therapeutic synergism was demonstrated. The optimum dose of methotrexate decreased and the optimum dose of teniposide increased in the combination as the interval between drugs was increased from 0 to 52 hours (12).

It is apparent that the concepts of drug activity and optimum dose have been more precisely defined from a statistical point of view using RSM and the understanding of drug interactions in combination treatments is improved. In this respect, the confidence region about the estimated optimum dose has come to be recognized as most informative.

#### *Drug Activity*

Until recently, the definition of drug activity in animal studies has been that the drug at some dose must produce an arbitrarily assigned increase in life span over untreated control animals. The criterion differs with different tumor systems and is set at a level that ideally avoids declaring drugs active as a result of spuriously positive results. The criteria developed are based on a large experience, and since they are set to avoid too many false-positives, it is likely that false-negatives occur. However, these techniques neither use all of the information available from the experimental data nor are they based on the use of an optimal level of treatment with the given agent. RSM has been used to develop a definition of activity based on the confidence interval about the location of the optimal treatment. If the $100(1-\alpha)\%$ confidence interval about the optimal treatment excludes the untreated control group, it can be concluded with $100(1-\alpha)\%$ confidence that treatment is better than no treatment and hence the drug can be claimed active (9). It should be noted that since the inference is based on the optimal level of treatment, it is reasonable to expect that the incidence of false-negatives would decline.

#### *Synergy and Related Terms*

Much has been written and many definitions advanced regarding drug interactions in combination treatment. A recent review covers many aspects of drug interactions (19). The terms synergism, additivity or zero interaction effect, and antagonism are best applied where the response in question increases as the dose increases. At a given level of response it is valid to apply these terms by determining the proportions of each of the component drugs required to produce the response in relation to the amount of each drug alone required to achieve the same response. If these same relationships held at different levels of response, there would be no problem with the definitions, but due to toxicity that is not usually the situation. More likely, with the types of responses, such as survival, that are important in cancer chemotherapy, a drug combination may be synergistic in one region of the treatment space and antagonistic in another. Thus, these terms lose their meaning with respect to a combination per se and have to be qualified with regard to not only the response sought but the region of the response surface involved.

The concept of therapeutic synergism has been useful throughout the years and is defined as the ability of a combination at optimum dose to achieve a result superior to the single drug or modality components at their optimum doses (20). Until recently, no statistically defensible method of claiming therapeutic synergism had been developed, but with RSM and the recently developed methods for calculating a confidence region around the estimated optimum dosage levels of the components of the combination it is possible to say that a combination of k drugs is therapeutically synergistic if the specified confidence region does not include a zero dose of any of the drugs in the combination (9).

### Adaptation of RSM to Cancer Chemotherapy

In applying RSM to cancer chemotherapy, it has been necessary to study and modify procedures previously used in industry and elsewhere. These efforts will be discussed according to the stages of a response surface analysis listed in the Historical Review Section.

#### *Experimental Design*

Animal experiments using drug combinations have usually employed factorial designs. Virtually all published studies use only two drugs, since factorial designs rapidly became prohibitive due to the large number of groups required by such designs (table 1). Two three-drug combinations reported by Goldin et al (21) utilized a ray design with 148 treatment groups each (*see* fig 1 for a graphic illustration of several types of designs that have been employed). In a ray design, each ratio of drugs, eg, a 1:1 mix or 2:1 mix, can be thought of as a single drug. Dose-response curves for these combinations can be presented, and in some respects the combination effects are simpler to present and hence to understand. A possible disadvantage is that treatment groups are clustered near the origin, which may be wasteful of resources.

The central composite design was developed as an alternative to the factorial design for use in fitting response surface models (22). The savings in the number of treatment groups relative to that required by the factorial design increases with the number of variables (table 1). In

TABLE 1.—No. of treatment groups utilized by various experimental designs*

| Type of experimental design | No. of drugs or treatment variables | | | | |
|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 |
| Factorial | 25 | 125 | 625 | 3125 | 15,625 |
| Central composite (with a single center point) | 9 | 15 | 25 | 43 | 77 |

* Calculations are based on the use of 5 treatment levels, which, in the case of factorial designs, usually include a 0 treatment level. For the central composite design, 0 doses are not included but 5 treatment levels of all variables are provided.

attempting to adapt a central composite design to the study of combinations of drugs, difficulty was experienced in predicting single-drug results. The central composite design requires no single-drug-treated animals or even untreated controls, and if these are not utilized the single-drug predictions may not be in close agreement with known results. Modification of the experimental design for biomedical applications seemed to be appropriate.

An important experimental design question is concerned with how to best allocate available resources. For example, given a fixed number of experimental subjects, is it more desirable to heavily replicate a few treatment groups than to increase the number of treatment groups at the expense of the number of experimental subjects available to each treatment combination? Stablein (23) simulated animal experiments involving a single drug and two-drug combinations in order to gain an indication of the answer to this question. The conclusions drawn from this study suggest that fixed resources should be distributed among many treatment points. In addition, given that the total sample is large enough, extensive replication of each treatment combination, contrary to present practice, is not necessary. It is important to realize that this recommendation is applicable when interest is directed toward estimating properties of the underlying dose-response relationship, eg, location of the optimal treatment, as opposed to making comparisons of the response at two different combinations.

In an additional simulation study, Stablein and Carter (24) studied the effect of censoring and grouping on the statistical properties of the estimates of the parameters associated with the underlying dose-response relationship, with the location of the optimal treatment, and with the response at the optimal treatment. An important finding of this study was that slight grouping of censored survival data was not associated with any meaningful deterioration in the estimation of the location of and response at the optimum treatment levels.

Another major aspect of efficient design in RSM is to place the treatment points in the region of the treatment space where optimum treatments would be expected to be found. This has led to the use of modified factorial designs for animal experiments in which low-dose and high-dose treatments are omitted. For a three-drug experiment, 49 treatment groups, as opposed to 125 for a full $5^3$ factorial design, have proven to be adequate (9), while four-drug experiments have used 177 groups instead of 625 groups (25). With two to three animals per treatment group and using the modified factorial designs it is feasible to perform three- and four-drug experiments. If time between treatment with two drugs is one of the variables, the ability to judge where the region of interest is located may be reduced and consequently more treatment groups are required (12).

There are implications for human studies. Defining the treatment region is important in human studies, since there are ethical reasons why ineffective or overly toxic treatments should not be given. If a treatment is perceived by the treating physician to be ineffective, it may not be pursued as vigorously as one perceived to be an adequate treatment. The Virginia Oncology group used

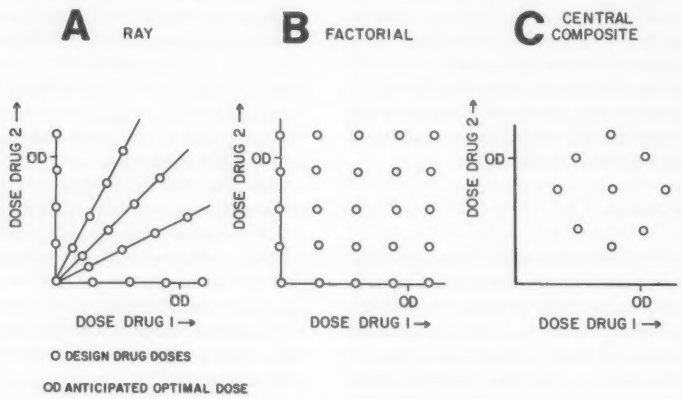


FIGURE 1.—Experimental design variations.

combinations of CTX and doxorubicin on a central composite design in a pilot study to treat squamous cell carcinoma of the lung (26). At the time the study was started in 1975, no known treatments were considered to be effective, so some very low-dose combinations were included. Low-dose combinations were quickly dropped by investigators, while higher-dose regimens were continued for longer periods of time. As a result of the simulation studies mentioned earlier, it would appear that a reasonable design for clinical studies would be to define carefully the region of interest and place a few patients on each of a large number of equally spaced treatment groups within the space. An example of such a design applied to the 5-FU, doxorubicin, and mitomycin (FAM) regimen is shown in figure 2. In this example, the 100% dose of 5-FU is 600 mg/m$^2$, the 100% dose of doxorubicin is 30 mg/m$^2$, and the 100% dose of mitomycin is 10 mg/m$^2$. Doses of the individual drug range from 67% to 133% of full dose, but overall doses (average of each of the three doses) range from 89% to 111% of full dose. It should be noted that it is much easier to find a suitable number of experimental groups when three or more variables are present than when two variables only are used.

#### *Model Selection and Parameter Estimation*

A property of the dose-response relationship is that with low levels of treatment the risk of failure is high. When treatment levels with effective agents are increased the risk of failure decreases until an optimum response is reached. As treatment levels increase from this point the risk of failure increases due to the toxicity of the treatment agents. The simplest model with these characteristics is the quadratic, which is typically used. It is important to note that it is not assumed that the true model is quadratic but rather that the quadratic model can adequately approximate the true relationship. In all cases, the method of maximum likelihood has been used to estimate the model parameters.

#### *Model Verification*

The $\chi^2$ test has been used to judge the goodness of fit when the logistic model is used. The response, instantaneous risk of failure, considered in Cox's proportional hazards model, is not directly observed and therefore the usual goodness of fit tests are not applicable in this situation. Developing tests for the goodness of fit of the proportional hazards model is the subject of ongoing research. Kay (27), Anderson (28), and Wei (29), among others, have reported on this. To gain an indication of the strength of the relationship between observed and predicted results, the groups can be ranked according to the estimated hazard and again by median survival time and the rank order correlation between these calculated. This correlation is somewhat analogous to the $R^2$ value reported in more standard regression analyses.

#### *Exploration of the Fitted Surface*

Once the model parameters have been determined and an adequate fit demonstrated, it is often informative to plot contours of constant biologic effect (isobols). Mathematical techniques have been developed to estimate the dosage levels associated with optimal response (30). Techniques for defining the confidence region about the optimum treatment (9) or the confidence interval about the response at the optimum treatment (10) have been described.

#### *Methods Not Requiring Assumption of the Mathematical Form of the Model*

Methods discussed thus far in this article have all required the assumption that a relatively simple mathematical model can adequately approximate the underlying dose-response relationship for a combination of therapeutic agents. Once the unknown constants which parameterize the mathematical relationship have been estimated, various procedures, as discussed herein, can be used to explore the relationship and estimate the optimal level of each agent used in the combination. As indicated, these procedures are applicable to the analysis of data from clinical trials. There is, however, another approach to designing a clinical trial to yield the optimal level of each drug in a combination. A major difference associated with this approach is that it does not require the assumption of the mathematical form of the underlying dose-response relationship. Box (2) suggested that evolutionary operation (EVOP) might be useful. EVOP is a technique developed for use in the chemical industry as a practical way of making the transition from the laboratory to full-scale production. Once in production, large changes in the production process could have a noticeable impact on the quality of the product and thus on the profitability of the operation. Hence, changes made in the process must be small to protect against the possibility of a noticeable deterioration in the quality of the product. This situation is similar to that faced by the chemotherapist who has developed an acceptable therapy as, for example, those currently used for lymphoma or Hodgkin's disease, but doubts that the combination is being used optimally. EVOP is a method for searching for better conditions using standard treatments which proceeds through a sequence of small changes in the operating conditions.

In a series of computer studies designed to simulate the clinical situation, Segreti (31) and Segreti et al (32) studied the use of an EVOP approach, among other methods, for estimating the optimal levels of each component in a combination of therapeutic agents. Since there is no optimization phase associated with the current method of conducting a clinical trial, it was not possible to determine the relative efficiency of the EVOP methods. However, these authors found, in each case con-

sidered, that the EVOP method moved in the direction of improved treatment until the improvement in response was of the same order of magnitude as the variability in the data. While there is clearly more work needed in this area, it would seem that such methods could ultimately find application in clinical studies and provide important information.

## Applications

### *Improved Ability to Conduct Multivariable Animal Studies*

There are few comprehensive three-variable studies in the literature and most of these have utilized response surface methodology (8,9,11-14,19). Approaches optimizing for a limited number of variables with the later addition of other variables while holding the original variables constant will most likely fail to arrive at optimal conditions (12).

### *Discovery of Favorable Drug Interactions*

Combinations showing therapeutic synergism as defined by RSM are being utilized clinically. These include the combination of 5-FU, teniposide, and mitomycin (9), and methotrexate and an epipodophyllotoxin with an interval between the two drugs (12). In both of these examples, the clinical use has been at the Medical College of Virginia and the Mid-Atlantic Oncology Group. Although the human data base for evaluating the accuracy of extrapolations from animal studies does not exist, it is important to recognize that the improved understanding of the dose-response relationship afforded by RSM is likely to provide a firmer basis for such extrapolation. Information about the drugs in a combination provided by use of RSM is listed below.

1. Relative doses in the combination versus optimal doses of the single drugs
2. Relative effect of the combination versus the effect of the single drugs
3. Regions of the treatment space where the effect is not statistically different from the optimum effect
4. Regions of the treatment space where the effect is statistically superior to single-drug treatments
5. The effect of tumor burden (if included in the experimental design) on optimum conditions
6. The effect of intervals between drug administration (if included in the experimental design)
7. Analysis for short-term versus long-term benefit

Only the first two listed above have been available by classical methods of analysis. The increased information associated with drug interactions provided by RSM

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | 400 | 400 | 400 | 400 | 400 | 400 | 400 | 400 | 400 | 400 | 533 | 533 |
| A | 20 | 26.7 | 26.7 | 33.3 | 33.3 | 33.3 | 40 | 40 | 40 | 40 | 20 | 20 |
| M | 13.3 | 11 | 13.3 | 9 | 11 | 13.3 | 6.7 | 9 | 11 | 13.3 | 11 | 13.3 |

| | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | 533 | 533 | 533 | 533 | 533 | 533 | 533 | 533 | 533 | 533 | 533 | 667 |
| A | 26.7 | 26.7 | 26.7 | 33.3 | 33.3 | 33.3 | 33.3 | 40 | 40 | 40 | 40 | 20 |
| M | 9 | 11 | 13.3 | 6.7 | 9 | 11 | 13.3 | 6.7 | 9 | 11 | 13.3 | 6.7 |

| | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | 667 | 667 | 667 | 667 | 667 | 667 | 667 | 667 | 667 | 667 | 667 | 667 |
| A | 20 | 20 | 20 | 26.7 | 26.7 | 26.7 | 26.7 | 33.3 | 33.3 | 33.3 | 40 | 40 |
| M | 9 | 11 | 13.3 | 6.7 | 9 | 11 | 13.3 | 6.7 | 9 | 11 | 6.7 | 9 |

| | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 |
|---|---|---|---|---|---|---|---|---|---|---|
| F | 800 | 800 | 800 | 800 | 800 | 800 | 800 | 800 | 800 | 800 |
| A | 20 | 20 | 20 | 20 | 26.7 | 26.7 | 26.7 | 33.3 | 33.3 | 40 |
| M | 6.7 | 9 | 11 | 13.3 | 6.7 | 9 | 11 | 6.7 | 9 | 6.7 |

FIGURE 2.—Proposed response surface experiment for the FAM regimen (doses in mg/m$^2$). This design is an expansion of the following fractions of the "full dose" of each drug, 600 mg/m$^2$ of 5-FU, 30 mg/m$^2$ of doxorubicin, and 10 mg/m$^2$ of mitomycin: 2/3, 8/9; 10/9, 4/3 with elimination of 18 groups getting low or high doses. The individual range of doses for drugs is large, with some groups getting twice as much drug as others. The total dose of drug ranges only from 89% to 111% of "full dose."

should be helpful in the difficult area of extrapolating from animal to clinical studies.

#### *Optimization With Constraints*

Thorough study of a treatment comprised of a combination of drugs requires that experiments be extended into the toxic dose range; otherwise the optimization procedure is likely to select a toxic dose. This problem has led to use of constraints which confine the process of optimum dose selection. A method has been developed where nonlethal toxic effects such as weight loss, leukopenia, or thrombocytopenia can be modeled as functions of drug doses. These models can be used as constraint functions to place limits on the selection of an optimum dose. This consideration in the analysis of toxicity as well as efficacy involves dual response surfaces and has the advantage of incorporating both the therapeutic and side effect analyses (33). Thus, it is possible to estimate the levels of treatment associated with optimal survival time given that the incidence of undesirable toxic effects is kept below a predetermined level.

The importance of this approach is that it provides a convenient way to extend these methods to clinical trials where deliberate use of toxic doses would be unethical. It has the additional advantage of allowing flexibility in dosage adjustment in individual patients because of the increased ability to understand the precise effects of dose on the generation of specific toxic effects. Results are presented graphically with a clarity previously unattained when more than two interacting variables contribute to the results (11). A method for estimating the confidence region about the constrained optimum combination has also been developed (34).

#### *Nonproportional Hazards Model*

The use of Cox's proportional hazards model assumes that throughout the time of the study, the risk of death of the animals in any treatment group remains constant. Unfortunately, for cytotoxic agents this may not be the case. From the time of injection of the cancer cells into a mouse until the day of treatment, all mice are subjected to a common risk of death. Upon treatment, however, mice receiving a moderately effective therapy should have a risk which is lower at all points in time relative to the controls. On the other hand, a very effective treatment may actually cause excessive risk at early time points due to drug toxicity which decreases as the animal survives the toxicity and benefits from the enhanced antitumor effect. Hence, the risk functions may not only be nonproportional, they may actually cross one another. This result has important clinical implications, as some treatments may be desirable for short-term success, while others improve the chance for long-term cures.

This nonconstancy of the risk function over time can be accounted for by including treatment-by-time interactions in Cox's model, resulting in the development of a nonproportional hazards model. Nonproportional hazards models have been shown to be appropriate in both preclinical (18) and clinical applications (35).

In the clinical application cited, a nonproportional hazards model was used to analyze a trial comparing chemotherapy alone to a combination of chemotherapy and radiation in locally advanced, nonresectable gastric carcinoma run by the Gastrointestinal Tumor Study Group. Survival curves estimated by the method of Kaplan and Meier (36) from the clinical data crossed one another. Early on, chemotherapy alone appeared to be the better treatment, while the combination gave better long-term survival. When this is the case it is possible that the log-rank test, a valuable, widely accepted method for analyzing survival data from comparative clinical trials, will lead to the conclusion of no difference when in fact a difference between the treatments exists. Analysis of these data by the proportional model, equivalent to the log-rank test, showed no difference in the relative risk of the two treatment groups. By including a treatment-by-time interaction term in Cox's model, it was shown that significant nonproportionality existed, and, further, that there was a significant difference in the risks of death experienced by the patients on the two treatment arms. Survival curves from the nonproportional model closely resembled the survival curves obtained by the method of Kaplan and Meier. Although the nonproportional model is sometimes required to approximately fit the data, the simpler proportional model may provide a reasonable result (18).

## CONCLUSION

It has been said that while all models are incorrect, some are informative. Taking this attitude, much can be gained from pursuing the RSM approach in both preclinical and clinical studies. Choice of a model is based on fit using as few terms as reasonable for the situation. Recognizing that model distortions can and do occur, it is important to find more flexible models, avoid extrapolations outside of the experimental region, and allow some leeway in the precise predictions of the model based on a knowledge of how model distortions affect results.

Current clinical studies do not provide any information about the location of the optimal treatment levels. This is a result of their design. By including additional treatment groups in such trials and using RSM in the analysis, one can gain such information with the result that one has a better basis for the selection of the next study to undertake. This may prove to be one of the most beneficial aspects of the application of this methodology to clinical studies.

It is apparent that RSM has been successfully applied to animal combination therapy studies. Currently, a transition to clinical studies is under way. It is our hope that this review will stimulate the imagination of other in-

vestigators to find further applications of this methodology in the biomedical sciences.

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# Alternation of Chemotherapy and Radiotherapy in Cancer Management. III. Results in Experimental Solid Tumor Systems and Their Relationship to Clinical Studies[1]

**W. B. Looney* and H. A. Hopkins[2]**

One of Frank Schabel's major concerns was the maximum utilization of therapeutic results obtained from studies using laboratory animal models for the improvement of cancer management. He pointed out that "laboratory and clinical oncologists commonly use different endpoints in reporting chemotherapeutic activity against solid tumors in animals and man. Usually, the laboratory oncologist starts drug treatment when tumors are grossly undetectable and reports drug activity on the basis of mean or median growth inhibition (cytostasis) in a group of tumor-bearing animals. In contrast, the medical oncologist requires tumor cell kill of several orders of magnitude, as evidenced by tumor regression due to drug treatment of grossly apparent tumors, before reporting drug activity against individual tumors in man" (1).

Studies designed to obtain experimental information relevant to the clinical management of cancer require a different approach than traditional basic studies designed to obtain precise information concerning a particular event or step in a metabolic pathway. In basic studies the event is made the limiting step in a chain of events to study how it may be modified. However, this event may be only one of many interacting or interrelated events which must be considered in cancer management. In basic research the concentration of studies to better understand one isolated event is acceptable from a scientific standpoint but the results obtained may not provide sufficient information to design a protocol which is effective from a clinical standpoint. In designing clinical protocols one integrates the known information and makes clinical judgments on the optimum way of proceeding with regard to tumor control and tumor cure. Marsoni and Wittes (2) summarized the problem with regard to polychemotherapy in these words: "Nevertheless, when a clinical investigator goes about putting together a combination regimen, he may be guided by evidence of synergism in laboratory models, but the final choices of agents, doses, schedules and sequencing are usually made on the basis of considerations other than hard science." These problems in directly utilizing the information obtained from basic research in no way detract from its value in better understanding the specific events related to drug or radiation action. The current limitations do not preclude the utilization of the *concepts* resulting from experimental studies to design more effective clinical protocols.

Clinical schedules for delivery of radiotherapy and chemotherapy have evolved independently. The underlying therapeutic strategy for the clinical utilization of chemotherapy has evolved from the necessity of using large doses separated by long time periods of 3–4 weeks to allow primarily for gastrointestinal tract and bone marrow recovery. The underlying therapeutic strategy for the clinical utilization of radiotherapy has evolved from the necessity of using small daily radiation doses to avoid major damage to the normal tissue surrounding the tumor. However, there is no reason to believe that these methods of delivery represent optimal therapies. Further experimental and clinical studies are needed to better utilize these two primary treatment modalities known to be effective against certain types of tumors.

## EXPERIMENTAL STUDIES

To date, the major portion of our experimental work on the interaction of chemotherapy and radiotherapy has been with cyclophosphamide (CP), with additional studies using 5-FU and doxorubicin, representatives of two other major classes of cancer chemotherapeutic agents (3–6). The proven value of CP, 5-FU, and doxorubicin in the management of a broad spectrum of cancer types makes them attractive agents to be used with radiation. The extensive amount of information that has accumulated in terms of their clinical effectiveness as well as toxicity makes these attractive agents to use in our continuing attempt to learn how to more effectively use chemotherapy and radiotherapy in tumor types responsive to both modalities. Improvements in the use of

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[2]Division of Radiobiology and Biophysics, University of Virginia School of Medicine, Charlottesville.

* *Reprint requests to:* Dr. W. B. Looney, Division of Radiobiology and Biophysics, University of Virginia School of Medicine, Charlottesville, VA 22908.

well-established chemotherapeutic agents by more effectively combining them with radiotherapy can be brought to clinical use much faster and with greater efficiency than can new agents. Information regarding optimal scheduling of these well-established cancer chemotherapeutic agents with radiation may also be useful in establishing the most effective way of using new chemotherapeutic agents as they are developed.

Frank Schabel believed that an animal model of the same tumor type was not necessary to obtain information relevant to the particular tumor in man. A therapeutic question resolved in a tumor model could often be applied to human tumors which were different. The Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, MD, initiated a program on preclinical combined chemotherapy and radiotherapy in 1976 with a number of institutions in this country (7,8). We were invited to participate in this program and the counsel of Frank Schabel through the years has been a major contribution to our own efforts in trying to utilize animal tumors for the improvement of cancer treatment. At the time of the initiation of the DCT program in preclinical treatment, we were working with Dr. Harold Morris and his associates at Howard University on a number of experimental hepatomas (9). Since a wealth of information was available on these hepatomas, it was natural to utilize these hepatomas for our collaborative efforts in the DCT program.

In the beginning phases of our experimental treatment program we evaluated the characteristics of six different tumors with different morphological, cell kinetic, growth (doubling times between 1 and 24 days), biochemical, and biological characteristics (9). From these six tumors we selected two for the radiotherapy and chemotherapy program. One tumor, H-4-II-E, is resistant to both radiation and the representatives of three major classes of chemotherapeutic agents we have evaluated, ie, 5-FU, doxorubicin, and CP; the other tumor, 3924A, is responsive to both radiation and these three chemotherapeutic agents. The experimental work presented in this review relates primarily to studies of the effects of radiation alone, CP alone, and the interaction of CP and radiation in hepatoma 3924A (3).

The effects of increasing single doses of CP alone were first determined. No cures were obtained when the dose was increased from 50 to 250 mg/kg (0.3–1.5 g/m$^2$). In rats, the intestinal mucosa recovers in approximately 6 days, but 10–12 days are required for recovery of bone marrow for single (LD10) doses of 5-FU, doxorubicin, and CP. This is approximately one-half the bone marrow recovery time in man of 17–21 days for comparable doses of cancer chemotherapeutic agents (3). There were no cures with sequential (LD10) doses of 150 mg/kg of CP every 11 days for two, three, four, and five courses (total doses, 300–750 mg/kg of CP). Three courses of 150 mg/kg of CP total on Days 0, 11, and 22 are optimal with regard to median survival in tumor-bearing rats (3).

Radiation was also ineffective in producing cures when used alone in 3924A. The effects of daily radiation schedules on tumors were evaluated over a total dose range of 3000–11,250 rad. This was carried out by maintaining a constant number of 30 fractions over the 1-month treatment period and increasing the fraction size from 100 to 150, 188, 250, and 375 rad daily. No complete responses (CR) or tumor cures occurred over the entire dose range. Therefore, conventional scheduling of either radiation alone or CP alone was unsuccessful in providing either complete tumor response or tumor cures.

It was found that by simply alternating large doses of radiation (in the order of 1500 rad) with the LD10 dose of CP on an 11-day cycle, cure rates of $\geq$ 50% could be obtained following three courses of induction therapy. Thus, an unsuccessful treatment protocol for induction therapy could be transformed into a successful protocol by alternating three courses of each modality rather than giving three or more courses of each modality alone. Despite the tremendous difference in weight of man and rat (70 vs 0.2 kg), only six courses of induction chemotherapy are conventionally given every 3–4 weeks in man, compared to three courses of alternating CP and radiation needed in the rat to obtain a cure rate of $\geq$ 50%.

We were able to employ large doses of radiation over a 2-day period by giving the radiation in multiple fractions per day (MFD). This markedly reduced the skin reaction for both acute and late effects and still permitted us to give the amount of radiation necessary to maintain cure rates $\geq$ 50% when alternated with chemotherapy (3).

Our studies to date have been primarily concerned with the effects of alternating chemotherapy and radiotherapy for induction therapy in which the primary objective is achieving CR of the primary tumor. The major clinical endpoints that we have been able to obtain in our experimental studies are shown in table 1 (10). In this experiment, three courses of CP were alternated with three courses of radiation. The schedules for MFD were devised by increasing the number of 250-rad fractions over a 2-day period. These 2-day schedules were given at 11-day intervals for three courses on Days 0 and 1, 11 and 12, and 22 and 23. CP (150 mg/kg) was given 1 day after each of the three radiation courses on Days 2, 13, and 24. Therefore, the total CP dose was held constant while the total radiation dose was varied from 750 to 7500 rad. Partial responses (PR) were obtained in the lower dose range with increasing numbers of CRs as the radiation dose was increased. The cure rate consistently increased with increasing doses. Cure rates of 30%, 50%, and 60% were obtained as the total radiation dose was increased from 4500 to 6000 and 7500 rad, respectively. The mean time to CR was in the order of 100 days and the median host survival was increased with increasing doses.

Lung metastases were prevalent in the groups with the lower radiation dose (range, 750–3000 rad). However, in the higher dose range (4500–7000 rad), metastatic dis-

TABLE 1.—Tumor cures and growth delay, median survival, and incidence of pulmon
with 1-day interval b

| Experiment No. | | Radiation Dose†, Day | Radiation Dose†, Day | CP, 150 mg/kg (Day) | Total dose (rad) |
|---|---|---|---|---|---|
| 786 | B | 250 × 1 | | | |
| | | 0 | | 1 | 750 |
| | | 11 | | 12 | |
| | | 22 | | 23 | |
| 786 | C | 250 × 1 | 250 × 1 | | |
| | | 0 | 1 | 2 | 1500 |
| | | 11 | 12 | 13 | |
| | | 22 | 23 | 24 | |
| 786 | D | 250 × 2 | 250 × 1 | | |
| | | 0 | 1 | 2 | 2250 |
| | | 11 | 12 | 13 | |
| | | 22 | 23 | 24 | |
| 784 | C | 250 × 2 | 250 × 2 | | |
| | | 0 | 1 | 2 | 3000 |
| | | 11 | 12 | 13 | |
| | | 22 | 23 | 24 | |
| 784 | E | 250 × 3 | 250 × 3 | | |
| | | 0 | 1 | 2 | 4500 |
| | | 11 | 12 | 13 | |
| | | 22 | 23 | 24 | |
| 784 | G | 250 × 4 | 250 × 4 | | |
| | | 0 | 1 | 2 | 6000 |
| | | 11 | 12 | 13 | |
| | | 22 | 23 | 24 | |
| 784 | I | 250 × 5 | 250 × 5 | | |
| | | 0 | 1 | 2 | 7500 |
| | | 11 | 12 | 13 | |
| | | 22 | 23 | 24 | |

* Ref 10.
†No. of rads × No. of fractions/day.
‡Ten animals in each group. Used in clinical context of no detectable evidence of tumor.
§Volume 50% less than initial treatment volume.
¶Time required for tumors to reach 8 times the volume at beginning of treatment.
* * Day of death.

f pulmonary metastases with increasing No. of radiation fractions (250 rad) combined with CP, interval between modalities*

| | Mean time to CR (days) | CR‡ | PS§ | Tumor cures | Time to $8V_0$¶ (days) | Median survival (days) | No. of animals with lung metastases |
|---|---|---|---|---|---|---|---|
| | — | 0 | 0 | 0 | 48.3 ± 2.2 | 64 | 3 |
| | — | 0 | 0 | 0 | 55.0 ± 2.6 | 83 | 8 |
| | — | 0 | 1 | 0 | 69.5 ± 3.9 | 68 | 1 |
| | — | 0 | 7 | 0 | 76.2 ± 2.4 | 101 | 7 |
| | 100 ± 4 | 3 | 7 | 3 | 116.3 ± 4.2 | >130** | 0 |
| | 106 ± 7 | 5 | 5 | 5 | 115.3 | >130** | 1 |
| | 89 ± 4 | 6 | 3 | 6 | — | >130** | 0 |

semination was eliminated with one exception. A major advantage of the early use of radiotherapy alternated with chemotherapy relates to the fact that chemotherapy has the potential for inhibiting the dissemination and growth of micrometastases in addition to acting in conjunction with localized radiotherapy on the primary tumor.

When CP was not given, no cures were obtained with daily radiation totaling 11,250 rad or with MFD radiation totaling 9000 rad in parallel experiments. The total dose for a 50% cure rate was 11,250 rad for daily fractions with CP, which was unacceptable with regard to host and normal tissue toxicity. The use of MFD schedules with CP reduced the total radiation dose needed for a 50% cure rate to 6000 rad, which was within the acceptable limits of toxicity for both modalities. This major reduction of the total radiation dose needed for a 50% cure rate from 11,250 rad with daily radiation schedules to 6000 rad by MFD schedules has obvious clinical ramifications. It suggests that major improvements in some human tumors may be possible by the more effective interaction of chemotherapy and radiotherapy.

Comparison of the first three experimental protocols in table 2 (A, B, and C) demonstrates the increased therapeutic effectiveness of alternating CP with radiation given as MFD over a short period of time compared to the alternation of CP with radiation given on a daily fraction schedule. The same total radiation dose of 7500 rad, the same fraction size (250 rad), and the same total dose of CP of 450 mg/kg were given in three courses over a period of approximately 1 month. The cure rate in Protocol A was 60%, in Protocol B was 70%, and in Protocol C was 0%.

There are three major advantages of Protocol B over Protocol C. First, there is a 7-day interval between the giving of radiation and CP in each course which allows recovery of acutely responding mucosal tissue following each modality. Second, the alternation of radiation and CP gives two treatments to the tumor but only one treatment to the bone marrow within each period of hematological recovery. This has a special advantage for CP since giving three courses 14 days apart exceeds the 10–12-day recovery period for the bone marrow. Third, giving the radiation as MFD and CP in three courses over a 36-day period results in a 70% cure rate, while the same total radiation given as daily fractions with the same total CP dose for the three courses over a 33-day period results in no tumor cures.

In table 2, Protocol D was carried out to determine the relative effectiveness of giving all of the 7500 rad as single 250-rad daily fractions prior to giving the three courses of CP every 11 days. No tumor cures or complete tumor responses occurred. However, the total treatment period was increased to 53 days.

Reversing the delivery of the two modalities by giving the three courses of CP every 11 days prior to the delivery of 7500 rad as 250-rad single daily fractions also resulted in no tumor cures and no complete tumor responses. The results obtained from this protocol underscore the importance of beginning treatment with both modalities as quickly as possible if successful induction therapy is to be realized.

These experimental results have been presented in a clinical context and are readily interpretable by clinicians involved in clinical cancer management. The results suggest that further experiments with comparable clinical parameters can become more useful in clinical protocol design. It should also result in the more rapid exchange of clinical and experimental results.

## CLINICAL STUDIES

Some pilot clinical studies are underway and others are being developed which will test the concepts that are evolving from our experimental and theoretical studies. Nine different clinical groups in this country and in Europe are beginning to assess the clinical effectiveness of alternating chemotherapy and radiotherapy in a number of types of tumors:

IA. *Small cell carcinoma of the lung*:
Professor M. Tubiana, MD, and associates, Institut Gustave-Roussy, Paris.
Howard Scher, MD, and associates, Memorial Sloan-Kettering Cancer Center, New York.

1B. *Non-small cell carcinoma of the lung*:
Giorgio Arcangeli, MD, and associates, Istituto Medico e di Ricerca Scientifica, Rome.

2. *Hodgkin's lymphoma*:
Richard T. Hoppe, MD, and associates, Divisions of Radiation Therapy and Medical Oncology, Stanford University Medical Center.

3. *Non-Hodgkin's lymphoma*:
Professor M. Tubiana, MD, and associates, Institut Gustave-Roussy, Paris, and the European Organization for Research on Treatment of Cancer (EORTC).

4. *Cancer of the head and neck*:
Michael Nissenbaum, MMBCh, and associates, University of Witwatersrand, Johannesburg, South Africa.

5. *Carcinoma of the ovary*:
Lester J. Peters, MD, and associates, University of Texas Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston.

6. *Carcinoma of the cervix*:
Professor M. Tubiana, MD, and associates, Institut Gustave-Roussy, Paris. Yosh Maruyama, MD, and associates, University of Kentucky, Lexington.

7. *Rectal/pancreas*:
Yosh Maruyama, MD, and associates, University of Kentucky, Lexington.

The experience of some of these clinical groups as it applies to alternating chemotherapy and radiotherapy in Hodgkin's disease, carcinoma of the lung, and head and neck cancer is given in the following sections.

### Hodgkin's Disease

The developing of clinical protocols for alternating chemotherapy and radiotherapy for the treatment of advanced Hodgkin's disease provides the prototype for the utilization of this approach for cancer management in general.

In an effort to provide maximum drug doses to patients, Kun et al (11) at the NCI treated a group of patients with advanced Hodgkin's disease with six cycles of mechlorethamine, vincristine, procarbazine, and prednisone (MOPP) followed by high-dose extended-field or total-nodal irradiation. These patients received doses of chemotherapy with an average of 81% of the calculated doses of mechlorethamine and procarbazine administered. Unfortunately, the combined modality therapy in this particular sequence appeared to have substantial morbidity and mortality.

Hoppe et al (12) concluded that it is not possible to combine sequential intensive chemotherapy and radiation therapy in the management of patients with advanced Hodgkin's disease and expect to be able to deliver optimal drug doses and optimal radiation therapy. The cumulative toxic effects, especially the marrow toxicity of both agents, are prohibitive. The doses of drugs which can be administered after completion of total-nodal irradiation are probably suboptimal. On the other hand, the morbidity of attempting to deliver total-nodal irradiation after the completion of six full cycles of chemotherapy is excessive. These results led Hoppe et al (12) from Stanford to protocols in which chemotherapy and radiotherapy could be used optimally. They suggested that the program of utilizing chemotherapy alternated with radiotherapy in stage III-IV Hodgkin's disease might be used as a model for treating patients with other malignancies.

The protocol for alternating radiotherapy with MOPP is shown in table 3. Split-course chemotherapy with radiotherapy was also used and that protocol is shown in table 4. In the split-course protocol all of the radiotherapy is given following the first two courses of chemotherapy and before the subsequent four courses of chemotherapy. Patients in the split-course group received an average of 65% of their calculated total drug doses, while those in the alternating-therapy group received 76.5%. Although the initial plan was for a simple split-course regimen, the alternating approach appeared to permit administration of even greater drug doses. The total drug doses tolerated by patients treated in the alternating fashion were similar to those reported for previously untreated patients. The total drug doses employed in the alternating group were greater than those reported in patients treated with involved-field radiation followed by adjuvant chemotherapy (13), and much greater than when MOPP was used as an adjuvant after total-nodal irradiation (14) or in the treatment of patients who had failed previous therapies (15).

The potential advantages of alternating the two modalities are summarized as follows. "The alternating technique should provide better control of occult extranodal disease and constitutional symptoms by minimizing the period of time during which the patient is off systemic therapy. The delayed irradiation of minimally or uninvolved lymphoid regions should result in less early cumulative marrow toxicity and the total drug doses which could be administered should be greater. Finally, the most extensively involved lymphoid regions could be irradiated early on during the course of therapy with full doses (4400 rad), while clinically uninvolved regions could be irradiated towards the end of therapy, with lower doses employed, if necessary, due to cumulative bone marrow toxicity" (12).

Although the Stanford group has not initiated a randomized trial in which patients with advanced Hodgkin's disease are treated with alternating chemotherapy and radiation versus other types of combined modality programs, a retrospective analysis of their experience was recently reported by Halberg et al (16). The median follow-up of survivors is 4.6 years (range, 1.3-13). The complete response rate is 87% for 49 patients with stage IIIB disease treated with alternating therapy. Among the complete responders, the 5-year survival rate is 98% and 96% are free from relapse. For both stage IIIB and IV, it appeared that the alternating chemotherapy and radiotherapy approach provided for the best survival and freedom from relapse. Based on its initial observations of superior drug doses with the alternating program and later results suggesting an excellent outcome for patients treated with alternating therapy, the Stanford group has adopted this approach as its standard treatment program for patients with stage IIIB disease and many patients with stage IV.

### Head and Neck Cancer

Tumors of the head and neck have been shown to be responsive to both chemotherapy and radiotherapy and, therefore, can be considered for different types of scheduling for the more effective utilization of these two treatment modalities.

Nissenbaum et al (17) at the University of Witwatersrand, Johannesburg, South Africa, found the response rate for patients with extensive head and neck cancer to be 69% for chemotherapy alternated with radiotherapy contrasted with 30% for radiotherapy alone and 23% for chemotherapy alone. The median survival for the combined modality treatment was 50 weeks contrasted with 24 weeks for the groups given either chemotherapy alone or radiotherapy alone.

The chemotherapy regimen consisted of cisplatin (20

Table 2.

EXPE

A. Alternating chemotherapy and radiotherapy given as
(Cyclophosphamide given 1 day after each of 3 cou

| Day in Cycle | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| 1st Cycle (Days 1-11) | R+ | R+ | C* | | | | |
| 2nd Cycle (Days 12-22) | R+ | R+ | C* | | | | |
| 3rd Cycle (Days 23-33) | R+ | R+ | C* | | | | |

+ Radiation given as 250 rads per fraction on days 1,2

* Cyclophosphamide, 150 mg/kg or 0.9 $g/m^2$, given on da

B. Alternating chemotherapy and radiotherapy given as
(Cyclophosphamide given 7 days after each of 3 cou

| Day in Cycle | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| 1st Cycle (Days 1-14) | R+ | R+ | | | | | |
| 2nd Cycle (Days 15-28) | R+ | R+ | | | | | |
| 3rd Cycle (Days 29-42) | R+ | R+ | | | | | |

+ Radiation given as 250 rads per fraction on days 1,2

* Cyclophosphamide, 150 mg/kg or 0.9 $g/m^2$, given on da

**Looney WB, Hopkins HA, Carter WH, Jr. Solid tumor r
alternate utilization of radiotherapy and chemothera

EXPERIMENTAL PROTOCOL**

iven as multiple fractions per day (MFD).**
3 courses of radiation,)

6 7 8 9 10 11

Tumor cure rate - 60%

ays 1,2; 12,13; 23,24. Total dose 7500 rads.
n on days 3, 14 and 25.

iven as multiple fractions per day (MFD).**
f 3 courses of radiation.)

6 7 8 9 10 11 12 13 14

C*

Tumor cure rate - 70%

C*

C*

ays 1,2; 15,16; 29,30. Total dose 7500 rads.
n on days 8, 22 and 36.

tumor models for the assessment of different treatment modalities. XXII. The
motherapy. Cancer 54:416-425, 1984.

C. Cyclophosphamide alternated with each of 3 courses of dail

| Day in Cycle | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| 1st Cycle (Days 1-11) | ←------- Radiation+ ---------- | | | | | | | | |
| 2nd Cycle (Days 12-22) | ←------- Radiation+ ---------- | | | | | | | | |
| 3rd Cycle (Days 23-33) | ←------- Radiation+ ---------- | | | | | | | | |

+ Radiation given as 250 rads/day; total dose 7500 rads.

* Cyclophosphamide, 150 mg/kg or 0.9 $g/m^2$, given on days 11, 2

D. Three courses of cyclophosphamide given after the completi

| Day in Cycle | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Days 1-11 | ←------- Radiation+ ---------- | | | | | | | | |
| Days 12-22 | ←------- Radiation+ ---------- | | | | | | | | |
| Days 23-33 | ←------- Radiation+ ----------→ | | | | | | | | ( |
| Days 34-44 | | | | | | | | | ( |
| Days 45-55 | | | | | | | | | ( |

+ Radiation given as 250 rads/day; total dose 7500 rads.

* Cyclophosphamide, 150 mg/kg or 0.9 $g/m^2$, given on days 31, 4

**Looney WB, Hopkins HA, Carter WH, Jr. Solid tumor models fo
A new approach to the more effective utilization of radiothe
Phys 11:2105-2117, 1985.

of daily radiation.**

8 9 10 11

----------→ C*

No tumors cured.

----------→ C*

----------→ C*

.

s 11, 22 and 33.

ompletion of 3 courses of daily radiation.

8 9 10 11

----------→

No tumors cured.

----------→

----→ C*

C*

C*

.

s 31, 42 and 53.

dels for the assessment of different treatment modalities. XXIII.
adiotherapy alternated with chemotherapy. Int J Radiat Oncol Biol

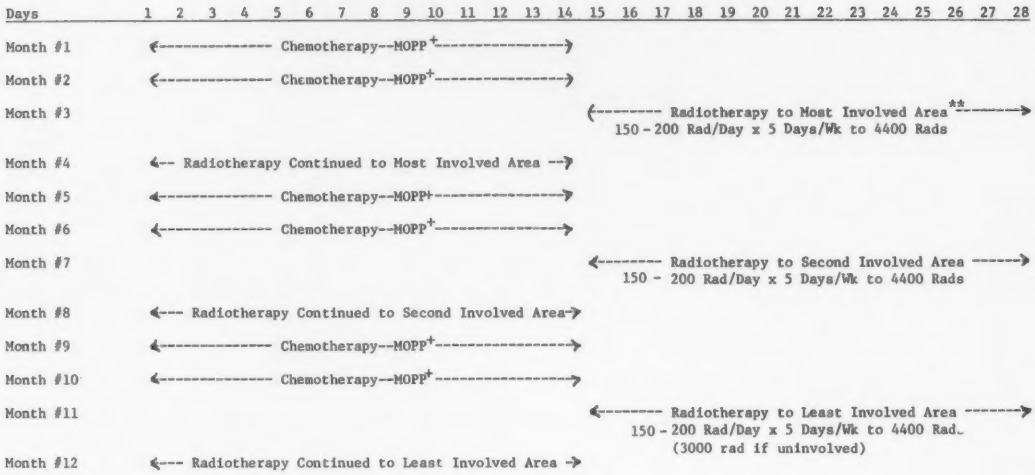
CLINICAL PROTOCOL

HODGKIN'S DISEASE

Table 3.

Alternating Chemotherapy and Radiotherapy

RT Hoppe, CS Portlock E Glatstein, SA Rosenberg, HS Kaplan*


Days
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Month #1
Chemotherapy--MOPP+
Month #2
Chemotherapy--MOPP+
Month #3
Radiotherapy to Most Involved Area**
150 - 200 Rad/Day x 5 Days/Wk to 4400 Rads
Month #4
Radiotherapy Continued to Most Involved Area
Month #5
Chemotherapy--MOPP+
Month #6
Chemotherapy--MOPP+
Month #7
Radiotherapy to Second Involved Area
150 - 200 Rad/Day x 5 Days/Wk to 4400 Rads
Month #8
Radiotherapy Continued to Second Involved Area
Month #9
Chemotherapy--MOPP+
Month #10
Chemotherapy--MOPP+
Month #11
Radiotherapy to Least Involved Area
150 - 200 Rad/Day x 5 Days/Wk to 4400 Rad.
(3000 rad if uninvolved)
Month #12
Radiotherapy Continued to Least Involved Area

*"Alternating chemotherapy and irradiation in the treatment of advanced Hodgkin's disease."

+ MOPP: 6 mg/m$^2$ Nitrogen Mustard days 1 and 8; 1 mg/m$^2$ Vincristine days 1 and 8; 100 mg/m$^2$ Procarbazine days 1 through 14; 40 mg/m$^2$ Prednisone days 1 through 14.

**Radiotherapy may start as early as day 1 of month 3 for this first treatment field.

mg/m$^2$ on Days 1, 2, and 3) and bleomycin (10 mg/m$^2$ iv on Days 1, 3, 5, and 7). This regimen was repeated at 4-week intervals. Three cycles of chemotherapy were given before assessing response. Maintenance therapy was given for another six cycles in responding patients or in those with stable disease. Two courses of radiation were given 8 days after the first and second courses of chemotherapy. The first course was given as two 240-rad fractions/day on Days 15-19, for a total dose of 2400 rad in 5 days. The second course of 2400 rad was given on Days 43-47 (table 5). Patients receiving radiation therapy had only local complications, which consisted of mucositis, skin erythema, and pigmentation. Most reactions were seen in the interval between radiation therapy courses and in no patient was it necessary to prolong the interval between treatments. The addition of chemotherapy did not significantly enhance the degree of local toxicity.

Fu (18) recently made an extensive review of the clinical use of concomitant chemotherapy and radiotherapy in the management of head and neck cancer. She considered that the toxicity of concomitant treatment in general outweighed its effectiveness. Further clinical studies directed toward optimally interacting chemotherapy and radiotherapy are warranted, not only for improvement of head and neck cancer but to provide more clinical information on how best to utilize these two primary treatment modalities.

Recent clinical studies of head and neck cancer provide a basis for further consideration of the relative effectiveness of alternating chemotherapy and intermittent radiotherapy compared with other methods of giving chemotherapy and radiotherapy. Al-Sarraf et al (19) at Wayne State University School of Medicine achieved a 55% CR rate in head and neck cancer in patients with extensive disease by infusion of 1 g/m$^2$ of 5-FU daily for 5 days with cisplatin. This is the highest response rate of any drug combination. The study of Wang et al (20) comparing radiation schedules of 160 rad twice daily and 180 rad daily illustrates how improved management of head and neck tumors may be realized by MFD alone. Marked improvement in local control rate at 36 months was observed for advanced tumors (T3 and T4) and with nodal disease by a twice a day radiation therapy program as compared to a conventional once a day every day radiation therapy program. There was a 59% actuarial control for patients given radiation twice a day compared to 26% for those given radiation every day.

The European Organization for Research on Treatment of Cancer studies on 179 patients with advanced head and neck tumors (21) demonstrated that increasing the

Table 4.

CLINICAL PROTOCOL

HODGKIN'S DISEASE

Split-Course Chemotherapy with Radiotherapy

RT Hoppe, CS Portlock E Glatstein, SA Rosenberg, HS Kaplan*

| Days | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 | 15 16 17 18 19 20 21 22 23 24 25 26 27 28 |
|---|---|---|
| Month #1 | ← Chemotherapy--MOPP+ → | |
| Month #2 | ← Chemotherapy--MOPP → | |
| Month #3 | | ← Radiotherapy to Most Involved Area → 150 - 200 Rad/Day x 5 Days/Wk to 4400 Rads |
| Month #4 | ← Radiotherapy Continued to Most Involved Area → | |
| Month #5 | | ← Radiotherapy to Remainder of Lymphoid System → 150 - 200 Rad/Day x 5 Days/Wk to 4400 Rads |
| Month #6 | ← Radiotherapy Continued to Lymphoid System → | |
| Month #7 | | |
| Month #8 | ← Chemotherapy--MOPP → | |
| Month #9 | ← Chemotherapy--MOPP → | |
| Month #10 | ← Chemotherapy--MOPP → | |
| Month #11 | ← Chemotherapy--MOPP → | |

*"Alternating chemotherapy and irradiation in the treatment of advanced Hodgkin's disease."

+ MOPP: 6 mg/m$^2$ Nitrogen Mustard days 1 and 8; 1 mg/m$^2$ Vincristine days 1 and 8; 100 mg/m$^2$ Procarbazine days 1 through 14; 40 mg/m$^2$ Prednisone days 1 through 14.

intensity of delivery of radiation by using MFD radiation schedules is effective in controlling tumor growth. Byfield et al (22) have shown that radiation can be effective when given with 5-FU infusion. They reported that 75% of 18 patients with stage IV disease achieved CR. Taylor et al (23) at Rush-Presbyterian-St. Luke's Medical Center combined these approaches by using 10-day intervals between sequential courses of treatment. Twenty-seven stage IV and three stage III cancers of the head and neck have been treated with promising results. Upon a median follow-up of 16 months (range, 6-35), only 11 (37%) have failed and eight (27%) have died. Of those patients who failed, six failed in distant sites, one developed a second head and neck primary, and only four (36% of all failures) failed regionally.

These five clinical studies using either radiotherapy alone, chemotherapy alone, chemotherapy alternated with radiotherapy, and radiotherapy and chemotherapy given together on an intermittent basis have all given promising initial results. These results point up the need for a systematic evaluation of these different approaches by prospective randomized clinical trials.

## Cancer of the Lung

### *Small Cell Carcinoma of the Lung (SCCL)*

Tubiana and associates at the Institut Gustave-Roussy, Paris, were first to use the concept of alternating chemotherapy and radiotherapy in the management of SCCL. The extensive clinical studies on the cell kinetic characteristics of human tumors made by the French group (24-36) provided much of the background information for this protocol. These studies have made a major contribution to the clinical utilization of chemotherapy and radiotherapy. In addition, these studies have greatly enhanced our understanding of the growth and cell kinetic characteristics of human tumors in general.

*Chemotherapy alternated with radiotherapy.*—Forty-five patients with limited disease were entered in a protocol from November 1981 to June 1983 (32). Patients were previously untreated and had histologically proven SCCL. All patients were < 70 years of age and their Karnofsky performance status (PS) was > 40%. Six cycles of induction chemotherapy (doxorubicin, 40 mg/m$^2$ on Day 1; etoposide, 75 mg/m$^2$ on Days 1-3; CP, 300 mg/m$^2$ on Days 3-6; and cisplatin, 100 mg/m$^2$ on Day 2) were given at monthly intervals. The first course of radiotherapy of 2000 rad given in 8 fractions over 12 days was alternated between the second and third courses of chemotherapy. The second course of 2000 rad was alternated between the third and fourth courses of chemotherapy. A third course of radiotherapy, 1500 rad in 6 fractions over 10 days, was alternated between the fourth and fifth courses of chemotherapy. Prophylactic cranial irradiation (3000 rad in 10 fractions over 14 days) was given during the first course of thoracic radiotherapy (table 6).

Table 5

TREATMENT OF ADVANCED HEAD AND NECK CANCER[+]

Alternating Multiple Fractions Per Day (MFD) Radiotherapy with Chemotherapy

M. Nissenbaum, S. Browde, W. Bezwoda, N. de Moor, D. Derman
Departments of Medicine and Radiation Therapy
University of the Witwatersrand, Johannesburg, South Africa

Group I

| Days | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 22 23 24 25 26 27 28 |
|---|---|
| Course I | ←Chemo*→ ←Radiation (240 rad fractions twice daily for 5 days) Total dose 2400 rad→ |
| | 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 41 52 53 54 55 56 |
| Course II | ←Chemo*→ ←Radiation Same as above.→ |
| | 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 |
| Course III | ←Chemo*→ |

*Chemotherapy at four-week intervals for a total of 9 courses:

Cis-Platinum 20 mg/m$^2$, days 1, 2, 3

Bleomycin 10 mg/m$^2$, days 1, 3, 5, 7

TREATMENT SCHEDULE

| Course | Chemotherapy | Radiation |
|---|---|---|
| First | 1,2,3,5,7 | 15 - 19 |
| Second | 29,30,31,33,35 | 43 - 47 |
| Third | 57,58,59,61,63 | ------- |

TREATMENT INTERVALS

| Course | C-R | R-C | C-C | R-R |
|---|---|---|---|---|
| First | 8 | --- | --- | --- |
| Second | 8 | 10 | 22 | 24 |
| Third | --- | 10 | 22 | --- |

+Nissenbaum M, Browde S, Bezwoda WR, de Moor NG, Derman DP. Treatment of advanced head and neck cancer: multiple daily dose fractionated radiation therapy and sequential multimodal treatment approach.

Patients achieving CR were given maintenance chemotherapy consisting of eight alternating cycles of two different drug regimens: *regimen 1*—mechlorethamine (5 mg/m$^2$ on Day 1), methotrexate (20 mg/m$^2$ on Days 1-2), and vincristine (1 mg/m$^2$ on Day 2); and *regimen 2*—etoposide (200 mg/m$^2$ on Days 1-2), lomustine (25-50 mg/m$^2$ on Day 2), and procarbazine (100 mg/m$^2$ on Days 3-10).

The principal dose-limiting toxic effect was hematological, which reached its maximal intensity after the fourth cycle of chemotherapy. Renal and neurological toxicity was slight (grade I) and reversible. No patient died of toxicity during the induction treatment. Thoracic radiotherapy was generally well-tolerated. Fibrosis within the radiation fields was recorded in 50% of patients, but symptoms such as cough and dyspnea on exertion were reported by only 7% of patients. Moderate esophagitis, necessitating medical treatment but no interruption of radiotherapy, was observed in 13% of patients; slight skin changes occurred in 6.5% and an infectious bronchopneumonia occurred in 17.7%.

Eight patients developed a local recurrence after CR (20%), most occurring within the radiation field. Two patients did not complete induction treatment because of progressive disease. Five patients were not controlled locally after the induction treatment, so the overall local failure rate was 29.5% (13 of 45 patients). Twenty-three patients developed distant metastases (51%). The mean time to the appearance of metastases was 16 months.

The high CR rate of 89% with acceptable toxicity demonstrates the effectiveness of relatively high-dose radiotherapy in three courses alternated with chemotherapy for induction therapy. The impact of this response on survival is shown in the 2- and 3-year relapse-free survival: 37% and 28%, respectively (table 7).

Brain metastases occurred in 15% of patients receiving prophylactic cranial irradiation (PCI) in this series. They consider that these results justify further specific study of the role of PCI in patients achieving CR. A randomized trial for patients achieving CR after alternating radiotherapy and chemotherapy is being initiated.

*Chemotherapy given sequentially with radiotherapy.*—Feld et al (37) at the Ontario Cancer Institute treated 163 patients with limited and 167 patients with extensive SCCL. Radiotherapy was given following six courses of chemotherapy. CP (900 mg/m$^2$), doxorubicin (45 mg/m$^2$),

Table 6

Protocol 1B
(031-004)

## SMALL CELL LUNG CARCINOMA+

M. Tubiana, R. Arriagada, J. M. Cosset
Institut Gustave-Roussy, Paris

| Days | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 29 29 30 |
|---|---|
| Month #1 First Cycle | ←——————→ Chemo* |
| Month #2 Second Cycle | ←——————→ Chemo* |
| | RT1** ←— 3000 rad to brain** (given as 300 rad ——→ 10 fractions/12 days fractions) |
| | RT1 ←— 2000 rad to tumor mass, mediastinum and ——→ 8 fractions/12 days supraclavicular nodes (given as four 250 rad fractions weekly) |
| Month #3 Third Cycle | ←——————→ Chemo* |
| | RT2 ←— 2000 rad to tumor mass, mediastinum and ——→ 8 fractions/12 days supraclavicular nodes (given as four 250 rad fractions weekly) |
| Month #4 Fourth Cycle | ←——————→ Chemo* |
| | RT3 ←—1500 rad to tumor mass, ——————→ 6 fractions/10 days mediastinum and supraclavicular nodes (given as four 250 rad fractions weekly) |
| Month #5 Fifth & Subsequent Cycles | ←——————→ Chemo* |

*Induction Chemotherapy

Doxorubicin, 40 mg/m$^2$ on day 1 — Cyclophosphamide, 300 mg/m$^2$ on days 3-6
VP 16213, 75 mg/m$^2$ on days 1-3 — Cis-platinum, 100 mg/m$^2$ on days 2

**Induction Radiotherapy

RT1, RT2 and RT3 are first second and third courses of radiotherapy

TREATMENT SCHEDULE
(Days after initiation of therapy)

| Course | Chemotherapy | Radiation |
|---|---|---|
| First | 1,2,3,4,5,6 | ---- |
| Second | 32,33,34,35,36,37 | 44 - 56 |
| Third | 63,64,65,66,67,68 | 75 - 86 |
| Fourth | 94,95,96,97,98,99 | 106 - 115 |

TREATMENT INTERVALS

| Course | C-R | R-C | C-C | R-R |
|---|---|---|---|---|
| First | --- | --- | --- | --- |
| Second | 7 | --- | 31 | --- |
| Third | 7 | 7 | 31 | 19 |
| Fourth | 7 | 7 | 31 | 19 |

+Tubiana M, Arriagada, R, Cosset JM. Sequencing of drugs and radiations. Cancer 55:2131-2139, 1985.

Arriagada R, et al. Alternating radiotherapy and chemotherapy schedules in small cell lung cancer, limited disease.

and vincristine (2 mg) (CAV) by bolus iv injection were given at 3-week intervals for a total of six courses unless the disease was clearly progressing. After six cycles of CAV chemotherapy, the pretreatment volume of intrathoracic tumor was irradiated in patients with both limited and extensive disease. Patients were treated with $^{60}$Co or a linear accelerator to a tumor dose of 2500 rad in 10 fractions over 2 weeks. Approximately 1 week after the third course of chemotherapy, responding patients underwent whole-brain irradiation. Patients were treated with 2000 rad in 5 fractions using a parallel pair of fields and a $^{60}$Co source.

Fifty-two percent of the patients with limited disease (LD) and 10% of the patients with extensive disease (ED) achieved CR. The median survival times for patients with LD and ED were 49 and 34 weeks, respectively. No maintenance chemotherapy was given (table 7). They concluded that prolonged maintenance chemotherapy for 1 year used in a previous study does not favorably influence outcome after intensive induction therapy for SCCL.

Although the use of PCI was found to reduce the frequency of brain metastases as the site of first relapse, detailed comparisons of response rates and survival showed no significant differences between the two study populations.

*Radiotherapy given concomitantly with chemotherapy.*—Lichter et al (38) of the NCI, Navy Medical Oncology Branch, used chemotherapy concomitantly with radiotherapy in the treatment of 74 patients with limited SCCL. All patients received CP (1500 mg/m$^2$), methotrexate (15 mg/m$^2$), and lomustine (100 mg/m$^2$) (CMC)/vincristine (1.4 mg/m$^2$), doxorubicin (50 mg/m$^2$), and procarbazine (100 mg/m$^2$) (VAP) chemotherapy. After Week 12 doses of CMC were reduced, and CMC/VAP were alternated for a total of 48 weeks of treatment.

Patients randomized to receive radiotherapy began their treatment within 3 days of the first chemotherapy cycle. The dosage of radiation (4000 rad in 15 treatments over 3 weeks) was chosen because their experience suggested that 3000 rad in 3 weeks was insufficient for reliable local tumor control. There was a CR rate of 49% in the 37 patients given chemotherapy alone contrasted with 78% of the patients given radiotherapy concomitantly with chemotherapy. The median disease-free interval was 36 weeks for the patients receiving chemotherapy and 68 weeks for the patients receiving chemotherapy and radiotherapy (table 7).

*Comparison of the different protocols.*—The results of the Institut Gustave-Roussy using alternating chemotherapy and radiotherapy are better than the results of

Table 7

INDUCTION THERAPY FOR LIMITED SMALL CELL CARCINOMA OF THE LUNG WITH CHEMOTHERAPY AND RADIOTHERAPY

| Method of Treatment and Clinical Group | Patient Number | Complete Response (percent) | Median Survival (weeks) | Relapse Free Survival (%) 2 yrs. | 3 yrs. |
|---|---|---|---|---|---|
| (A) ALTERNATION OF CHEMOTHERAPY & RADIOTHERAPY | 45 | 89 | 84 | 37 | 28 |
| Tubiana, et al. (Paris) | | | | | |
| (B) SEQUENTIAL CHEMOTHERAPY & RADIOTHERAPY (All chemotherapy given before radiotherapy) | 153 | 52 | 49 | 19 | 10* |
| Feld, et al. (Toronto) | | | | | |
| (C) CHEMOTHERAPY & RADIOTHERAPY GIVEN CONCOMITANTLY | 37 | 78 | | | |
| Lichtner, et al. (Bethesda) | | | | | |

*Projected

giving all six courses of chemotherapy prior to giving radiotherapy at the Ontario Cancer Institute. There was a CR rate of 89% in the study of Arriagada et al (32) compared to 52% in the study of Feld et al (37) (table 7). The median survival was 84 weeks compared with 49 weeks, respectively. Furthermore, the relapse-free survival at 2 years was 37% in the French study compared to 19% in the Canadian study. The relapse-free survival at 3 years was 28% compared to a projected 10% in the study of Feld et al (37). These clinical studies are comparable to our experimental results using alternating chemotherapy and radiotherapy versus the administration of all of the chemotherapy prior to giving radiotherapy. We were able to obtain a cure rate of 60%-70% by alternating radiation and chemotherapy, contrasted with no tumor cures if all three courses of one treatment modality were given prior to giving three courses of the other modality (table 2). Giving all of the induction chemotherapy first would eliminate the possibility of radiotherapy reducing the buildup of chemotherapeutically resistant tumor cells. The small total radiation dose of 2500 rad appears to be less effective than the 4500 and 5500 rad used in the two French pilot studies.

The results of Lichter et al (38) at the NCI are intermediate between the French and Canadian studies. The CR rate was 78% (table 7), and the median disease-free interval was 68 weeks. The better results of the study by Lichter et al compared to the study by Feld et al probably reflect giving the radiation early with the chemotherapy as well as a larger total radiation dose (4000 vs 2500 rad). The major disadvantage of the study by Lichter et al is the increased toxicity that occurs with giving the two modalities concurrently. This toxicity will, in all probability, limit the total radiotherapy and chemotherapy doses that can be given during induction therapy. The ability of the study by Arriagada et al (32) to temporally separate the delivery of the two modalities with better therapeutic results overcomes one of the major drawbacks to the more effective use of these primary treatment modalities. The alternating of the two modalities allows for twice the normal tissue recovery time for each modality. This offers the potential for major increases of the total doses of both modalities during induction therapy as well as the potential for increases in the effectiveness of control of the primary tumor and its metastatic dissemination.

Future clinical studies planned at the Institut Gustave-Roussy include increasing the total radiation dose from 5500 to 6500 rad and intensifying the delivery of radiation doses by giving MFD intermittently and alternately with chemotherapy. The projected clinical studies are consistent with the results of our experimental studies optimizing the utilization of alternate chemotherapy and radiotherapy in induction therapy. The increase in the total radiation dose from 5500 and 6500 rad has the potential for further reduction of the 20% local recurrence

rate. Intensifying the intermittent radiation schedule by the use of MFD has the potential for more effective interaction of radiotherapy and chemotherapy on the primary tumor as well as reducing the 50% incidence of distant metastases. Our experience in experimental studies suggests that the planned clinical studies for improving the management of SCCL should be one of the more effective approaches for better control of the primary tumor as well as better control of metastatic dissemination.

A number of clinical and cooperative groups are evaluating protocols for carcinoma of the lung, but alternating chemotherapy and radiotherapy has not been attempted in these protocols. Results of clinical studies using combined chemotherapy and radiotherapy have been reported by Mira and Livingston (39), Greco et al (40), Morstyn et al (41), Komaki et al (42), Perez et al (43), and Duncan (44).

Bleehan (45) has recently summarized the results of five groups using a combination of three chemotherapy agents with radiotherapy in the treatment of 227 patients with limited SCCL. There was a CR rate of 60% and a median survival which ranged from 48 to 74 weeks.

### *Non-Small Cell Carcinoma of the Lung*

A number of clinical and experimental studies have utilized MFD radiation treatments in which radiation is given two to three times daily based on the assumption that recovery of normal tissue from radiation fractions given 4 or more hours apart is faster than tumor recovery (46–54). MFD permits delivery of the total radiation dose over a short time span and has the potential for being more effective than conventional daily clinical fractionation schemes in combined radiotherapy and chemotherapy protocols.

Attempts have recently been made to improve local control for a number of tumor types by utilizing MFD schedules. It appears from the clinical data so far obtained that MFD schedules show results comparable to the best results achieved with conventional daily schedules, suggesting that greater flexibility of patient management in clinical radiotherapy may be possible without a major loss of effectiveness. Such schedules allow a large radiation dose to be delivered in a shorter than conventional time period without prohibitive late effects. Barendsen (51) and Thames et al (52) have recently discussed the radiobiological basis for these modified schedules.

Arcangeli et al (49) at the Istituto Medico e di Ricerca Scientifica, Rome, have carried out a pilot study in 31 patients with stage IV non-small cell carcinoma of the lung since March 1982. One of the primary findings of the pilot study was the ability to give one course of 1200 rad of radiation as MFD during each of the first 2 weeks of treatment alternated with one course of chemotherapy, with most patients having very mild or no side effects. Radiotherapy was given as three 200-rad fractions on Days 1 and 2, 8 and 9, 22 and 23, and 29 and 30, for a total dose of 4800 rad over a 30- to 32-day treatment period. A three-drug combination of CP (400 mg/m$^2$), doxorubicin (17 mg/m$^2$), and methotrexate (15 mg/m$^2$) was alternated with radiotherapy on Days 3 and 24 and repeated thereafter every 21 days (table 8). The overall response rate in this study was 68%, which is a response much higher than other results in extensive disease. The median survival was 35 weeks for the entire group of patients and 44 and 15 weeks for the responders and nonresponders, respectively.

One of the primary findings of this pilot study was the ability to give one course of 1200 rad of radiation as multiple fractions per day during each of the first 2 weeks of treatment alternated with one course of chemotherapy, with most patients having very mild or no side effects. Giving multiple daily fractions 4 hours apart permits the delivery of a large amount of radiation over a short time period (ie, 1–2 days) within the limits of normal tissue toxicity. Increasing the recovery time of radiotherapy by alternation with chemotherapy offers the possibility of increasing the total radiation dose beyond the upper limits now considered feasible by conventional radiation schedules for induction therapy.

The major deterrent to further improvement in the management of non-small cell carcinoma of the lung using alternating chemotherapy and radiotherapy relates to the lack of effective chemotherapy agents for this group of cancers. However, the information obtained by Arcangeli et al is useful for the design of SCCL protocols in which both treatment modalities are effective. This information can also be used in the testing of new chemotherapeutic agents which may eventually prove to be effective in the management of non-small cell carcinoma of the lung.

## Implications for Future Clinical Studies

Tubiana et al (29–32) recently summarized the status of 25 years of combined chemotherapy and radiotherapy. They indicated that it is traditional to distinguish between two kinds of goals in the combined approach. The first is spatial cooperation in which the objectives of each of the two kinds of therapy are different. For example, radiotherapy tries to sterilize the original tumor, while chemotherapy tries to destroy the metastases which have spread throughout the body. The second goal is to treat a single tumor with radiotherapy and chemotherapy. At first, radiotherapy and chemotherapy seem complementary. In reality, the distinction between the two categories of objectives outlined above is theoretical. It is more important to be aware of the cumulative effects of the two treatments in normal tissues because the success or failure of the combined treatment depends on the tolerance of the tissues. Thus, the real distinction is between alternating and simultaneously administered radiotherapy and chemotherapy.

Concomitant combined radiotherapy and chemother-

Table 8

CLINICAL PROTOCOL (ORIGINAL--NUMBER 1)

NON-SMALL CELL LUNG CARCINOMA

G. Arcangeli, R. Righini, C. Nervi, A. Guerra, D. Tirindelli, Istituto Medico e di Ricerca Scientifica, Rome
H. Hopkins, W. Looney, Div. Radiobiology & Biophysics, University of Virginia School of Medicine, Charlottesville, VA

| First Cycle Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 600 rads 200x3 | 600 rads 200x3 | C** | | | | | 600 rads 200x3 | 600* rads 200x3 | | | | | | | | | | | | |

| Second Cycle Days | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 600 rads 200x3 | 600 rads 200x3 | C** | | | | | 600 rads 200x3 | 600* rads 200x3 | | | | | | | | | | | | |

Continue chemotherapy at 21-day intervals to toxicity or progression of disease.

**Induction: Mtx 15 mg/m$^2$
CTX 0.5 g/m$^2$
Adr 17 mg/m$^2$

*Radiotherapy: 3 fractions per day of 200 rad each
2400 rad in 9 days
4800 rad in 30 days

Treatment Schedule
(Days after Initiation of Therapy)

| Course | Radiation | Chemo. | Radiation |
|---|---|---|---|
| First | 1, 2 | 3 | 8, 9 |
| Second | 22, 23 | 24 | 29, 30 |

(Days between treatment)

| Course | R-C | C-R | C-C | R-R |
|---|---|---|---|---|
| First | 1 | 5 | 21 | 6 |
| Second | 1 | 1 | — | 6 |

apy is less well-tolerated by normal tissues than sequential administration. Furthermore, since chemotherapy has to be continued for long periods to be effective, the benefits of concomitant administration appear to be minimal in relation to its inconveniences. For alternating treatment, a time interval of 1 week between the two therapies can reduce cumulative toxic effects on normal tissues. Moreover, it is preferable to administer each of them as early as possible to reduce the risk of development of resistant tumor cells.

Tubiana et al (29-32) described the advantages of alternating chemotherapy and radiotherapy as follows. (*a*) Chemotherapy is not delayed and may begin early. Moreover, the interval between two cycles is not modified, which avoids the risk of allowing the metastases to increase in volume between the two series of chemotherapy. (*b*) The effects of radiotherapy and chemotherapy are cumulative in the tumoral tissues, since the intervals between the two therapies are too short to give the tumor time to grow unless, of course, the tumor is completely resistant to radiotherapy and chemotherapy. (*c*) Toxic effects in normal tissues are minimal because the critical tissues are not the same for each type of therapy. They conclude that the alternation of chemotherapy and radiotherapy is one of the solid approaches to further progress in cancer treatment.

It may be possible to intensify radiation and chemotherapy delivery during the initial phases of induction therapy with acceptable toxicity and improved tumor control. Our experimental studies have shown that the first two courses of induction therapy must be effective in reducing tumor volume if our treatment is to be successful. However, controlled trials will be necessary to definitely establish the response rate. Further clinical evaluation of the effects of intermittent MFD radiotherapy with and without chemotherapy is also needed to assess the contribution of chemotherapy in the combined radiotherapy and chemotherapy protocol. However, the findings of these pilot clinical studies of non-small cell lung carcinoma (49) indicate that it is feasible to proceed with further clinical evaluation of this approach.

If more intensive treatment is possible, this may be accomplished by: (*a*) increasing the amount of radiotherapy given, (*b*) increasing the amount of chemotherapy

Table 9

SUGGESTED REVISED PROTOCOL (NUMBER 2)

CLINICAL PROTOCOL - NON-SMALL CELL LUNG CARCINOMA

G. Arcangeli, R. Righini, C. Nervi, A. Guerra, D. Tirindelli, Istituto Medico e di Ricerca Scientifica, Rome
H. Hopkins, W. Looney, Division of Radiobiology & Biophysics, University of Virginia School of Medicine, Charlottesville, VA

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| First Course Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| | 600 rad 200x3 | 600 rad 200x3 | 600* rad 200x3 | | | | | | | | | C** | | | | | | | | | |
| Second Course Days | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
| | 600 rad 200x3 | 600 rad 200x3 | 600* rad 200x3 | | | | | | | | | C** | | | | | | | | | |
| Third Course Days | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 51 | 62 | 63 |
| | 600 rad 200x3 | 600 rad 200x3 | 600* rad 200x3 | | | | | | | | | C** | | | | | | | | | |

Continue chemotherapy at 21-day intervals to toxicity or progression of disease.

**Induction: chemotherapy, to be determined.

*Radiotherapy: 3 fractions per day of 200 rad each
3600 rad in 24 days
5400 rad in 45 days

Treatment Schedule
(Days after initiation of therapy)

| Course | Radiation | Chemo. |
|---|---|---|
| First | 1, 2, 3 | 12 |
| Second | 22, 23, 24 | 33 |
| Third | 43, 44, 45 | 54 |

Time Interval
(Days between treatment)

| Course | R-C | C-R | C-C | R-R |
|---|---|---|---|---|
| First | 9 | -- | -- | -- |
| Second | 9 | 10 | 21 | 19 |
| Third | 9 | 10 | 21 | 19 |

given, or (c) decreasing the time between radiation courses. The following discussion illustrates how one might proceed to further evaluate how more effective scheduling of radiotherapy alternated with chemotherapy might be accomplished.

This suggested revision in the original protocol (table 8) is based on the promising therapeutic results with mild or no toxicity and on discussions with Dr. Arcangeli concerning the possibility of increasing the total radiation dose by giving 3 fractions of radiation on each of 3 days instead of 2 days (table 9). The increased time between radiotherapy followed by chemotherapy and chemotherapy followed by radiotherapy is to permit larger doses of radiotherapy and chemotherapy to be given within acceptable limits of toxicity. The data of Tubiana et al (30) on SCCL indicate that a 7-day separation is feasible. Our experimental data indicate that temporal separation of this magnitude of delivery of the two modalities is possible without a major loss of therapeutic effectiveness. Results on pulmonary metastases in man indicate that acceleration of growth begins on Day 8 after treatment (28). Our experimental results demonstrate acceleration of tumor growth beginning about the same time after after either radiation or CP. The superior therapeutic results with alternation of radiation and CP may be interpreted as one modality making the other modality more effective. Larger doses of more effective chemotherapy would also test the strategy of Goldie and Coldman of alternating the treatment regimen at every cycle to minimize the risk of developing doubly resistant tumor cells.

An increase in the amount of chemotherapy (CAM) given in each course could be considered since mild toxicity was found in the initial Italian study. The other possibility would be to change the chemotherapy to CAP in view of the response rate of 47% after two cycles of CAP found in the study of Fram et al (55) at the Dana-Farber Cancer Institute.

The primary purpose of the protocol in table 10 is to illustrate how the total radiation dose might be increased to $\geq$ 6000 rad within the framework of Protocols 1 and 2. The 7-day rest interval is based on the clinical results of Tubiana et al and our experimental results. Our experimental results have shown that the first two courses are critical to the success (tumor cure rates $\geq$ 50%) or failure of treatment. This protocol is designed to deliver as much chemotherapy and radiotherapy as possible within ac-

Table 10

SUGGESTED REVISED PROTOCOL (NUMBER 3)

CLINICAL PROTOCOL - NON-SMALL CELL LUNG CARCINOMA

G. Arcangeli, R. Righini, C. Nervi, A. Guerra, D. Tirindelli, Istituto Medico e di Ricerca Scientifica, Rome
H. Hopkins, W. Looney, Division of Radiobiology & Biophysics, University of Virginia School of Medicine, Charlottesville, VA

| First Cycle Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 600 rad 200x3 | 600 rad 200x3 | 600* rad 200x3 | | | | | | | C** | | | | | | | 600 rad 200x3 | 600 rad 200x3 | 600* rad 200x3 | | |

| Second Cycle Days | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 600 rad 200x3 | 600 rad 200x3 | 600* rad 200x3 | | | | | | | C** | | | | | | | 600 rad 200x3 | 600 rad 200x3 |

Continue chemotherapy at 21-day intervals to toxicity or progression of disease.

**Induction: Chemotherapy--to be determined.

*Radiotherapy: 3 fractions per day of 200 rad each
3600 rad in 19 days
5400 rad in 28 days
6600 rad in 43 days

Treatment Schedule
(Days after initiation of therapy)

| Course | Radiation | Chemo | Radiation |
|---|---|---|---|
| First | 1, 2, 3 | 10 | 17, 18, 19 |
| Second | 26, 27, 28 | 35 | 42, 43 |

Time Interval
(Days between treatment)

| Course | R-C | C-R | C-C | R-R |
|---|---|---|---|---|
| First | 7 | 7 | 25 | 14 |
| Second | 7 | 7 | -- | 14 |

ceptable limits of toxicity during the first two courses.

The pilot clinical study of Tubiana et al on lung cancer using the alternation of chemotherapy and radiotherapy has given results greater than or equal to conventional methods of using combined chemotherapy and radiotherapy. Temporal separation of the two modalities was possible, with promising therapeutic effectiveness. Temporal separation of the two modalities also avoids the excessive toxicity of concurrent administration of the two modalities, overcoming one of the major obstacles in the past clinical use of combined radiotherapy and chemotherapy. One of the major needs is to determine the tolerance levels of the lung when time is allowed for recovery between successive courses of radiation given intermittently (either as daily or MFD schedules) and to compare this with the effects of conventional daily radiation schedules. Recovery between courses should permit higher radiation doses to be given, thereby increasing the therapeutic ratio. Equally important is the need to establish tolerance levels for daily and MFD radiation given intermittently and alternated with CP. The clinical studies indicate the feasibility of giving higher total doses of each modality than used in conventional scheduling. This underscores the need for quantitative information on the upper limits of lung tolerance with radiation and chemotherapy given intermittently, allowing for recovery between treatment courses. This need is further emphasized by plans of Tubiana et al to increase the total radiation dose from 5500 to 6500 rad for induction therapy in the treatment of SCCL.

The current and projected clinical studies in the treatment of SCCL at the Institut Gustave-Roussy parallel our current and projected experimental studies designed to more effectively alternate chemotherapy and radiotherapy. They also illustrate how the continuous interchange of clinical and experimental information can be effectively utilized in the continued improvement of cancer management of SCCL.

## DISCUSSION

The ability to rationally design experimental protocols to more effectively utilize chemotherapy and radiotherapy provides a basis for designing clinical protocols for tumors which are responsive to both treatment modalities. The utilizing of chemotherapeutic agents of proven value and established toxicity has major advantages in ongoing efforts in learning how to optimally utilize chemotherapy and radiotherapy.

One of the most clinically relevant concepts that has evolved from our experimental studies is the superiority of alternating CP and radiotherapy in three courses of induction therapy. A cure rate of $\geq 50\%$ was obtained in experimental solid tumors with this protocol with acceptable toxicity to the host as opposed to no cures from three or more courses of either modality alone at total doses

which exceeded the permissible levels of damage to the host (3).

New methods of radiation scheduling in which the rate of delivery of radiation can be intensified by giving MFD have further demonstrated the potential for clinical exploitation of alternating chemotherapy and radiotherapy. In 3924A a total dose of 11,250 rad of daily radiation alternated with three courses of CP was needed to produce a 50% cure rate. A total dose of only 6000 rad of radiation given as MFD over a 2-day period and alternated with three courses of CP was needed to produce a 50% cure rate (3).

A major advantage of the early use of radiotherapy alternated with chemotherapy relates to the fact that chemotherapy has the potential for inhibiting the dissemination and growth of micrometastases in addition to acting in conjunction with localized radiotherapy on the primary tumor. In the experimental tumor 3924A, lung metastases were prevalent in the groups with the lower radiation dose range (combined with CP). However, in the higher dose range metastatic dissemination was eliminated with one exception (table 1).

The major clinical parameters used in the assessment of response of induction therapy have been obtained in these experimental studies. Partial tumor response, complete tumor response, mean time to CR, tumor regrowth following induction therapy, median survival, and development and control of metastatic dissemination of the primary tumor have all been assessed (table 1). To date, extensive studies have been carried out with one chemotherapeutic agent (CP) in one solid tumor model with radiation. These results demonstrate that the major questions of concern to the clinician in the design of clinical protocols can be addressed in experimental solid tumor systems. The results can be expressed in clinical terms that are interpretable by both the clinical radiotherapist and chemotherapist. Further experimental studies with different chemotherapeutic agents and radiotherapy can provide experimental information which can be useful to clinicians involved in protocol design in different tumor types.

The promising therapeutic results and host tolerance in pilot clinical studies which alternate chemotherapy and radiotherapy and show response rates and median survival times in excess of those for more conventional utilization of chemotherapy and radiotherapy warrant further and more extensive evaluation in controlled clinical trials. The promising results for Hodgkin's disease, head and neck cancer, and small and non-small cell lung cancer in four different cancer treatment centers underscore the potential for improvement of cancer treatment in these and other tumor types by more effectively utilizing these two primary treatment modalities. Results of both the clinical and experimental studies also underscore the validity of these concepts for use in future studies in tumors that are responsive to both chemotherapy and radiotherapy.

The alternation of chemotherapy and radiotherapy has the advantage of greater flexibility in scheduling chemotherapy and radiotherapy. Should the initial studies prove to be effective, then further increased effectiveness might be realized by increasing the chemotherapy doses given, reducing the time interval between the delivery of chemotherapy and radiotherapy, or a combination of these variables.

The use of mathematical models in the design and interpretation of results of clinical and experimental cancer treatment has contributed to major advances in the clinical management of cancer and to an understanding of some of the mechanisms involved. The concept of log cell kill by chemotherapeutic agents as developed by Skipper and Schabel (56) at the Southern Research Institute is an excellent illustration of how concepts which evolve in experimental tumor models find application in clinical protocol design. Testing and application of the concept of log cell kill in both animal models and man led to the realization that some cells within a seemingly sensitive tumor were resistant to treatment. Therapeutic strategies were developed to overcome various types of resistance: early and sequential treatments to overcome kinetic resistance and combinations of agents to prevent repopulation of the tumor by cells permanently resistant to a single agent. Goldie and Coldman (57-59) have developed a mathematical model for relating the drug sensitivity of tumors to their spontaneous mutation rate. With two effective therapies, their therapeutic strategy which minimizes the risk of development of doubly resistant tumor cells for a wide variety of cases is one that alternates the treatment regimen at every cycle. The value of this approach appears to be substantiated by the experience of Bonadonna (60), who has alternated the use of two non-cross-resistant drug combinations for treatment of Hodgkin's disease.

The concept formulated by Goldie et al is consistent with conclusions from our experimental data, although we approached the problem by exploring different treatment schedules. The experimental and theoretical bases of the two approaches are considered in more detail in the first two papers of this series (61,62). Our experimental results have demonstrated that alternating three courses of radiation and CP is superior to either modality alone in that we have transformed an untreatable situation to a treatable one, since no cures could be obtained by either modality alone within the limits of normal tissue toxicity. The superiority of alternating chemotherapy and radiotherapy may be related to factors other than reducing resistance. Some of the many factors which may contribute to the success of alternating chemotherapy and radiotherapy over conventional schedules are: (*a*) an accelerated growth of the tumor occurs, starting about 7 days after the preceding therapeutic insult, which may increase the effectiveness of the subsequent treatment, since increased therapeutic sensitivity is realized in more rapidly proliferating tumors; (*b*) the possibility of chemo-

therapy inhibiting the repair of potential lethal damage (occurring in hours), and (*c*) the inhibition of repair of chromosome damage (occurring in days).

Recently Weichselbaum and Little (63) suggested that repair of potentially lethal damage may correlate with clinical radiocurability. Several classes of agents have been described which appear to inhibit or suppress this recovery phenomenon. However, little is known about the effects of chemotherapeutic agents on recovery of tumor cells from radiation-induced damage. The potential for therapeutic exploitation of these differences in repair of potential lethal damage has been discussed in more detail in a previous paper in this series (61).

To date, we have evaluated only one chemotherapeutic agent at a time (primarily CP) in schedules with radiotherapy. In general, polychemotherapy is more effective than single-agent chemotherapy in clinical tumors which are responsive to chemotherapy. Therefore, the potential is greater for multidrug combinations with radiotherapy than for single drugs.

Delivery of radiotherapy in schedules which combine chemotherapy and radiotherapy needs special consideration. As Rubin (64) has stated: "There are no longer set radiation tolerance doses *per se* for normal tissues and organs in this combined modality era." The mathematical model developed by Ellis (65,66) to relate the effects of total dose, fraction size, and duration of radiation delivery was used for a number of years in both clinical and experimental radiotherapy to express the effects on both normal tissue and tumors. The expression for normal tissue was as follows: rets, Rad equivalent therapy units = total dose $\times$ $F^{-0.24}$ $\times$ $T^{-0.11}$, where F = number of fractions and T = total treatment in days (65). Another formula for equivalent units for tumors omitted the factor of time and is based on the assumption of a lack of recovery of the tumor during treatment: rens, Rad equivalent neoplasm units = total dose $\times$ $F^{-0.24}$ (66).

It has become clear from both clinical and laboratory data summarized by Thames et al (52) and Fowler (67-69) that there is a consistent difference between early and late responding tissues in their response to changing fractionation. As pointed out by Fowler (69), only multi-, small-dose experiments can provide data relevant to fractionated treatments in radiotherapy. When fewer and larger dose fractions are given, late reactions are more severe even though early reactions are matched by an appropriate adjustment in total dose.

The linear-quadratic model for cell survival following irradiation is frequently used to describe multifraction isoeffect data by the formula ln (response) = $-n(\alpha d + \beta d^2)$, where d is the dose per fraction, n is the number of fractions, and $\alpha$ and $\beta$ are obtained from the intercept and slope, respectively, of the straight line of the appropriate data plot. There is now increasing interest in estimating the quantity $\alpha/\beta$, since there appears to be a consistent difference in the $\alpha/\beta$ ratios for late-responding versus acutely responding tissues. If the linear-quadratic dose-response curve is appropriate (69), it should be possible to establish a total dose and dose per fraction for each schedule of radiation treatment which will just be tolerated for both early and late responding normal tissues. Tumors are considered as acutely responding tissues, thus, the importance of obtaining the maximum tolerated level of acute damage. However, there is meager information on the question of either experimental or clinical response of tumors acting as acutely responding tissues. Additional clinical and experimental studies on different tumor types are needed to determine the relationship of the response of the tumor to that of early and late responding tissue for radiotherapy alone as well as chemotherapy alone or combined chemotherapy and radiotherapy.

Thus, in both experimental and clinical chemotherapy and radiotherapy new mathematical models are being developed in continuing efforts to more accurately relate the relative efficiency of cancer treatment on the tumor in relation to host toxicity for different treatment strategies. The major emphasis of these mathematical models has been related to either chemotherapy or radiotherapy. Results of both clinical and experimental studies combining chemotherapy and radiotherapy indicate that mathematical models will be needed to effectively relate different forms of combined modality treatment to different therapeutic strategies.

Another approach to the more effective utilization of chemotherapy and radiotherapy utilizes the endpoints of treatment response and toxicity to estimate optimal treatment schedules (70). The major advantage of this method of analysis is that dose-response surfaces, indicating optimum treatment levels, can be estimated from discrete experimental results. It therefore has the capabilities of determining the relative risk of failure associated with single-agent chemotherapy with radiation as well as multiagent chemotherapy and radiotherapy by considering the constraints of normal tissue reaction and survival after treatment so that the rate of failure does not exceed a specific value. These treatment levels can be estimated from response-surface analysis and therefore are not dependent on predicting assumptions as in the mathematical models discussed in the previous section (70).

The different models for the assessment of therapeutic response are not mutually exclusive. Collaborative studies are continuing with Goldie and Coldman et al at the Cancer Control Agency of British Columbia and with Carter et al at the Medical College of Virginia. These two basically different methods of analysis are being used to analyze the results of our experimental cancer treatment program and provide valuable information about the design of future experiments to answer key questions concerning the alternation of chemotherapy and radiotherapy.

Frei and Canellos (71) reviewed the effects of dose and dose rate of cancer chemotherapeutic agents in experi-

mental in vivo systems and various clinical situations. They point out that the dose-response curve for most chemotherapeutic agents is steep. Although the dose-response curve may be linear for cytokinetically homogeneous experimental tumors, it deviates from linearity as a result of tumor heterogeneity. Thus, for highly sensitive tumors, the curve is very steep and generally linear; whereas, for relatively insensitive tumors, dose may have little effect on response. These observations in general apply to the clinic. When dose has been a randomized variable, a dose-response curve is generally evident in sensitive tumors.

The relationship between dose and local cure of Hodgkin's disease by radiotherapy has been discussed by Kaplan (72,73). If 3600-4000 rad are delivered in 5 weeks, the local cure is > 90%. The cure rate is only 50%-60% for one-half of this dose. Thus, there is a critical dose that must be reached to achieve cure, and any significant reduction from that dose compromises the cure rate substantially.

Frei and Canellos (71) indicate that in adjuvant chemotherapy situations in which prolonged, disease-free survival is improved, the dose-response curve would also appear to be quite steep. Conservative treatment in this setting may compromise the cure rate, and they propose to maintain full-dose therapy and consider reducing the total duration of treatment.

Tolerance to normal tissue presents limits beyond which the doses of chemotherapy and radiotherapy cannot be increased. In 3924A neither CP nor radiation can be used alone to obtain cure within the tolerable doses to normal tissues. However, by alternating the two treatments, the impact of treatment on the tumor is sufficient to obtain cure without exceeding the tolerance of normal tissues. At this time it is not known whether the therapeutic advantage from alternating chemotherapy and radiotherapy results from (*a*) a kinetic effect of increased tumor cell kill by a second agent during the accelerated growth which occurs as the tumor recovers from the first agent, (*b*) preventing the development of resistance by using two non-cross-resistant therapies, (*c*) a spatial effect resulting from additive effects of the two therapies on the tumor but not for critical normal tissues, or (*d*) an interaction of the two modalities, as, for example, an alteration in the extent of potential lethal damage repair in tumors treated with CP, or even from some combination of these and other factors. However, the advantage does exist in the experimental system and, as shown above, in those tumors of man where the concept has been applied.

The rapid development in our better understanding of the molecular basis of cancer should eventually lead to therapeutic exploitation of these recent developments. However, these new methods of treatment such as monoclonal antibodies, labeled antibodies, lymphokines, etc, may need to be combined in most instances with either or both chemotherapy and radiotherapy to be of maximum clinical usefulness. This underscores the need to learn how to use chemotherapy and radiotherapy optimally in clinical cancer management. It is necessary to be able to use these two primary treatment modalities optimally to achieve the most effective interaction of new treatment methods with them.

## CONCLUDING REMARKS

We have been able to rationally design experimental protocols in a solid tumor system (3924A) which has permitted us to more effectively use one established and effective cancer chemotherapeutic agent (CP) with radiation. These experimental findings have been presented in a clinical context and are readily interpretable by clinicians involved in clinical cancer management. The results indicate that further experimental studies with comparable clinical parameters can continue to provide information which can be useful in designing clinical protocols for more effectively alternating chemotherapy and radiotherapy in cancer management. The ability to obtain results in experimental solid tumor systems comparable to results obtained in clinical studies should facilitate the more rapid exchange of clinical and experimental results. We have used three different clinical tumors (Hodgkin's disease, head and neck cancer, and lung cancer) to illustrate how the data from one experimental solid tumor can be used to obtain experimental information useful in clinical protocol design. This illustrates Frank Schabel's tenet that the same tumor type is not necessary to obtain clinically relevant information.

We have shown that marked improvement in tumor cure rates can be achieved during induction therapy by alternating chemotherapy and radiotherapy compared to the repeated treatment of either modality alone. These results have also demonstrated the critical nature of the timing of chemotherapy and radiotherapy delivery in therapeutic success or failure. We have also shown that the use of multiple daily fractions of radiation given intermittently is much more effective when alternated with chemotherapy. In addition, the MFD radiation schedules can eliminate pulmonary metastases during induction therapy contrasted with only a reduction in pulmonary metastases when daily radiation schedules are alternated with chemotherapy. It is therefore possible to obtain in experimental solid tumor systems information relevant to two major clinical problems: (*a*) the control and cure of the primary tumor, and (*b*) its relationship to the control and elimination of metastatic dissemination. We plan to extend these completed experimental studies with alternating CP and radiation to three other drugs of proven clinical value in the treatment of a broad spectrum of human tumors: doxorubicin, 5-FU, and cisplatin. Since most clinical protocols employ more than one drug, we plan to initiate studies to determine the optimum interaction of a combination of two drugs alone (ie, without radiation). When the optimum interaction is determined, then it will be alternated with radiation to

determine the optimum interaction when alternated with radiation. This will provide additional information on the cross-sensitivity of alternating these drugs with radiation as we have done with CP and radiation. We believe that extending our studies to include these other drugs is justified by (*a*) the success with alternating chemotherapy and radiotherapy experimentally; (*b*) its promising clinical application to treatment of lung cancer, head and neck cancer, and Hodgkin's disease; (*c*) the clinical studies planned or in progress in this country and Europe alternating chemotherapy and radiotherapy in the treatment of cancer of the cervix, ovary, analrectal region, pancreas, and non-Hodgkin's lymphoma; and (*d*) the continued clinical problem of development of drug resistance for these and other extensively used cancer chemotherapeutic agents.

One of the primary needs for future clinical improvement in the treatment of lung cancer is to determine the tolerance levels of the lung when time is allowed for recovery between successive courses of radiation and/or chemotherapy given intermittently. Specific objectives for further experimental studies are: (*a*) to determine the effects of daily fractions of radiation alone and with concurrent CP on rat lung using doses per fraction and total doses which are within the current and projected clinical range (3000–7500 rad) for radiotherapy of lung cancer; and (*b*) to determine the isoeffective doses in rat lung for radiation schedules in which the radiation is given intermittently as daily fractions or as MFD, alone or alternately with CP, as compared with the continuous daily radiation schedules. These studies will provide a multiparametric evaluation of both the acute and late effects of treatment on lung structure and function.

At a workshop sponsored by the Cancer Therapy Evaluation Program of the DCT on June 19, 1985, we and representatives from cooperative groups and cancer treatment programs throughout the United States discussed the tumor types which would best lend themselves to clinical testing of the concept of alternating chemotherapy and radiotherapy. A major part of the workshop was devoted to the discussion of protocols for both SCCL and non-small cell lung cancer. There are almost no data relevant to the tolerance limits for the lung with the new and more effective treatment schedules of alternating chemotherapy and radiotherapy. Information from our projected studies should permit clinical groups to more fully exploit the therapeutic potential of alternating chemotherapy and radiotherapy in lung cancer management. Plans were made at the June 19 meeting of the DCT workshop to meet one or two times per year to update and exchange clinical and experimental results of studies in this country and Europe.

DeVita (74,75) has summarized concepts relating to the development of resistance to chemotherapy. He concludes that it is obvious that more effective anticancer drugs are still required to maximally exploit our current knowledge in cancer chemotherapy; but it also seems likely that more can be achieved with current drugs. The complicated nature of the assumptions of the Goldie-Coldman model, when individual variance in specific tumors, drugs, and clinical circumstances is added, makes it likely, however, that these advantages will ultimately accrue only with the help of computer modeling in advance of treatment, to design idealized protocols or individualized treatments for certain diseases as suggested by Goldie and Coldman and Skipper.

The use of well-established chemotherapeutic agents and improvement in their more effective utilization with radiotherapy can be brought to clinical use more rapidly and with greater efficiency than the use of new agents which would entail great periods of time in the exploration of their effectiveness. Our experimental solid tumor system can be used to answer some of the questions raised by these clinical studies and aid in designing alternative clinical protocols. The continuous interchange of results of experimental and clinical programs offers one of the more promising approaches for the more effective treatment of solid tumors in man using these two primary means of cancer management. New therapeutic approaches to improving cancer treatment will most likely be coordinated with either chemotherapy or radiotherapy or combined chemotherapy and radiotherapy. The promising therapeutic results of clinical studies in this country and Europe underscore the validity of the concepts that have evolved from experimental studies as well as the potential for improvement of cancer management by more effectively utilizing chemotherapy alternated with radiotherapy.

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# The Norton-Simon Hypothesis Revisited

**Larry Norton[1,*] and Richard Simon[2]**

By the mid-1970s, clinical oncology was facing an enigma. Patients with a wide variety of neoplasms—leukemias, lymphomas, small-cell lung cancer, and ovarian carcinoma, to name some—were regularly achieving complete remission on combination chemotherapy. However, many of these remissions were not durable. Patients would relapse, often with cancer histologically identical to the original, and, surprisingly, with retained biochemical drug sensitivity. This latter aspect was perplexing, for relapse could only be the result of two possibilities: inadequate killing of drug-sensitive cells or, alternatively, the presence of drug-resistant cells. The relapsing patients did not seem, at the time, to be undertreated, for they routinely had received at least two cycles of marrow-suppressive chemotherapy after the total clinical disappearance of their cancers. It would have been reasonable to assume that a few cells had escaped treatment because of biochemical resistance to the drugs employed. Examples of this latter phenomenon were well known in the laboratory (1). Yet, in the clinic, as rigorously documented in advanced Hodgkin's disease (2), the reapplication of the original chemotherapy could frequently reinduce complete remission. Clearly, the vast majority of cells at first relapse could not be biochemically resistant, or they would not have responded to the second application of the original drugs. At subsequent relapses, true cellular resistance would dominate: this was then evident in an inability to attain significant disease regression. Yet, the question posed by the first relapse remained unresolved: how could biochemically sensitive cells so completely escape intensive induction therapy? Could some factor besides drug resistance explain the discrepancy between remission and cure rates?

This enigma seemed related to an evolving view of postsurgical adjuvant chemotherapy. Active agents and combinations had been identified for stage IV breast cancer, and these therapies had been applied immediately after mastectomy for patients with stage II disease. Prevailing theory suggested that such an approach should be highly effective for several reasons (3). The microscopic tumor was smaller, and therefore fewer cells had to be eradicated. Small tumors often had a high percentage of mitotically active cells, and therefore a high percentage of cells were metabolically vulnerable to drugs which disrupt the mitotic cycle. Although preliminary results from early trials were indeed enticing (4), by the mid-1970s, some disturbing trends were apparent (5). While relapse rates between treated patients and controls were indeed different, it was clear that a high percentage of the micrometastatic tumors found in the postsurgical setting were not being easily cured by adjuvant chemotherapy. This was especially evident in survival curves for postmenopausal patients. Merely increasing the number of drugs in the adjuvant regimen did not necessarily improve results. In addition, patients with larger tumor burdens (ie, more positive axillary lymph nodes) seemed to be receiving more benefit than those with lower burdens. This suggested that the relationship between tumor growth and curability was not as straightforward as theory indicated. In sum, the effect of adjuvant chemotherapy was real, but, at least in the randomized studies, not as great as anticipated. Why was treatment less curative than had been reasoned?

In light of these problems, we set out to examine the relationship between tumor size and response to therapy. Our approach involved a relatively uncomplicated mathematical model, chosen to provide explanations for some of the clinical anomalies observed. The model also suggested approaches for improving therapeutic results using existing cytotoxic agents. This paper will briefly review these ideas and examine relevant clinical results generated in the 7 years since the model was first published.

## The Log-Kill and Norton-Simon Models

In the mid-1970s, the dominant model relating tumor size to responsiveness to therapy was the log-kill model, developed by Skipper, Schabel, and coworkers at the Southern Research Institute (8). Following subcurative treatment, the regrowth of murine leukemia L1210 was observed to trace a curve indistinguishable from untreated cells. Hence, it was possible to estimate the number of cells killed by a therapy measurement of prolongation of survival (6,7). This task was facilitated by the fact that the growth of L1210 was very closely approximated

[1]Mount Sinai School of Medicine, New York, NY.
[2]Biometric Research Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.
[*]*Reprint requests to:* Larry Norton, MD, Department of Neoplastic Diseases, Mount Sinai Medical Center, Fifth Ave and 100th St, New York, NY 10029.

by the exponential growth curve. In exponential growth, the percentage of cells engaged in mitosis stays constant over time. Hence, the whole tumor increases by a constant fraction over time. Say it takes x days for a tumor to increase by a factor of 10, which is called a 1-log increase. Over any x-day interval, the tumor will increase by 1 log, regardless of the starting time or initial cell number. Skipper observed that when a given number of L1210 cells in vivo were treated with a fixed dose and schedule of chemotherapy, a certain fixed survival prolongation was achieved. This was consistent with the death of a certain number of cells. Remarkably, the extent of the survival prolongation was the same regardless of the original number of L1210 cells at the start of treatment. This could only be explained if the therapy killed a certain percentage of the cells present, not a fixed number of cells.

The log-kill model can be stated mathematically in the following way:

$$dN(t)/dt = GF(N) \cdot N(t) - K \cdot L(t) \cdot N(t) \quad [1]$$

When N(t) is the number of tumor cells at time t, the left-hand side of the equation represents the rate of change of N(t) with time, L(t) represents the effective level of therapy at time t (functionally related to the dose level and schedule of drug administration), and K is a constant. GF(N) represents the instantaneous growth fraction for a tumor of size N. This is defined as dN(t)/dt divided by N(t) for an untreated tumor and is related to the percentage of cells engaged in mitosis (13). For exponential growth, GF(N) is constant. Equation 1 states that the rate of change in number of tumor cells is determined by the rate at which new cells are added to the population minus the rate at which existing cells are killed and removed. New cells are added at a rate determined by the product of GF(N) and N(t). The rate of cell kill is proportional to the cell number N, with a proportionality that is determined by the level of therapy L. Reference 9 contains more details about this model.

An implication of the log-kill model [Equation 1 with GF(N) constant] is that large tumors will always appear to regress much more rapidly than small tumors. We found that this was not consistent with many clinical observations. Large tumors often regress more slowly than tumors of equivalent histology but smaller size. Hence, advanced tumor size is of adverse prognostic significance in many diseases curable at small volumes (10,11). This effect may be related to genetically stable biochemical drug resistance (12), but could also be explained on purely kinetic grounds. While the assumption of exponential growth is reasonable for murine leukemia, most experimental solid neoplasms grow by Gompertzian kinetics, in which GF(N) decreases with increasing N (14–17). This pattern has been rigorously documented in human multiple myeloma (18) and testicular carcinoma (19). Since most anticancer drugs attack mitotic cells (20), the fraction of dividing cells may be important in determining the responsiveness of a tumor. We expressed this concept in the following equation:

$$dN(t)/dt = GF(N) \cdot N(t) - K_1 \cdot L(t) \cdot GF(N) \cdot N(t) \quad [2]$$

This model is equivalent to Equation 1, except that the rate of cell kill is now proportional not only to the level of therapy L, but also to the instantaneous growth fraction at that time. For exponentially growing tumors, GF is constant regardless of tumor size, so the two models are identical. For other patterns of tumor growth, the models differ. An example is Gompertzian growth, where Equation 1 would predict that the rate of regression would decrease with shrinking tumor size and that a "cure volume" may never be reached. By Equation 2, the rate of regression would also decrease, but to a lesser extent. Nevertheless, the overall rate at which the tumor shrinks toward a "cure volume" would be shallow for small-volume Gompertzian cancers. While substantial experimental data support Equation 2 (9,21–27), the therapeutic implications of this model and of our generalization of the log-kill model (Equation 1) are actually quite similar. These implications are the following:

1. The level of therapy necessary to cause a tumor regression or even a "complete clinical remission" may not be sufficient to eradicate the tumor.

2. The level of therapy necessary to cause the regression of large tumors, eg, in the metastatic setting, may not be sufficient to cure small tumors in the adjuvant setting.

3. In the setting of complete remission, the level of therapy which prolongs the durations of the remission may not cause cures, even if given indefinitely.

All of these implications hold, even in the absence of biochemically resistant cells. These points can be intuitively appreciated by noting that for tumors with Gompertzian-type growth curves, the rate of regrowth increases as the tumor shrinks. Hence, the level of therapy adequate to initiate a regression may not be sufficient to sustain the regression and produce cure.

These points do not necessarily predict universal failure, for in some individuals the rate of regression of small-volume disease in response to conventional therapy will be sufficient to achieve a volume that precludes later regrowth. Nor does this analysis deny the importance of true biochemical resistance which could be important in many instances. However, even in the absence of biochemical resistance, all of the above implications hold. The result is that some cancers may be difficult to cure entirely on kinetic grounds.

In both models, the rate of shrinkage of a tumor is only partially dependent on intrinsic kinetic factors. The other major determinant is the level of therapy. By the mid-1970s, it was already clear that higher doses of an anticancer agent would usually cause greater rates of regression than lower doses against similar tumors. Simi-

larly, more frequent administration could sustain a certain rate of regression for a longer period, and thereby accomplish more overall tumor volume regression. Subsequent analysis of clinical data (28,29) has supported these conclusions, and specific experiments have confirmed a rising relationship between clinical-range dose and response for many chemotherapies (23,30). We therefore suggested that one way of combating the slowing rate of regression of a tumor as it shrinks in response to therapy was to increase the intensity of treatment as the tumor became smaller. This could be accomplished in two ways. The first method was to use a certain combination of agents as an induction, and then increase the dose level of the whole combination or individual components. This could be termed an *intensification*. The second method was to abandon the original agents once remission was obtained, and use new agents, perhaps in combination, but always at an aggressive dose schedule. This would be a *cross-over intensification*. We recognized that this latter approach would also address the issue of true biochemical resistance. This was known to be an important problem for some patients on first relapse and for most on subsequent relapses. Specifically noted was that the tendency for low-dose "maintenance" programs, using drugs at suboptimal levels after initial chemotherapeutic or surgical induction, was a particularly poor approach to postremission therapy (31).

The concept of intensification was formulated with respect to the issue of toxicity as well as antitumor effect. For both models it can be shown mathematically that the smallest resulting tumor size for a given total level of therapy [integral of L(t)] is accomplished if the entire therapy is given over as short a time period as possible, ie, as induction treatment. Because of toxicity, however, this is generally impossible. Consequently, in 1977 we recommended the concept of using an induction to reduce the tumor volume to a size eradicable with a clinically tolerable intensification. This was a practical compromise. The concept was to intensify therapy as soon as possible, as much as possible, and for as long as possible, rather than to prolong a reduced level of therapy in the then-standard "maintenance" approach. The likelihood of success for an intensification plan depends upon the absence of biochemically resistant cells and the degree to which therapy can be intensified.

The concept of intensification, with or without cross-over, was later labeled the Norton-Simon hypothesis, to distinguish it from the Norton-Simon model. The hypothesis did not assert that intensification *must* be better than other designs. The model merely indicated that such plans *might* be better, if the tendency for slow rate of regression at small volumes could be overcome by sheer force of dose. It was certainly recognized that it was mathematically possible that for some tumors, no level of intensity achievable in the clinic could accomplish this goal. It was suggested, however, that late intensification was worthy of clinical trial, to determine if the dose levels that were feasible were sufficiently efficacious. Such trials could then be examined with respect to kinetic models, which could prove useful in interpreting the clinical results.

## Review of Clinical Trials

No well-controlled clinical trials have yet employed the concept of pure intensification therapy. To some extent, this is because of an interest in administering a large number of drugs in an effort to overcome biochemical resistance. Some promising regimens designed primarily to overcome biochemical resistance, however, may in fact achieve their advantage due to the principles described above. This is particularly so in programs which use a cross-over from one combination of drugs to another. Consolidation of a complete remission with a new combination may, in some cases, be effective because of increased cell kill of tumor cells that remain sensitive to the drugs used for induction. Because biochemical resistance is sometimes the major problem, it is certainly sensible to design regimens that attempt to circumvent both forms of resistance. Success with such regimens may, however, be difficult to interpret in terms of biological principles. Randomized trials with appropriate control groups are necessary to adequately determine whether one can successfully improve outcome by eradicating sensitive cells through intensification. In large part, these trials have not been performed. We will, however, review the indirect and preliminary experience obtained that is relevant to the concept of intensification therapy.

Hodgkin's disease is a classic example of a tumor that can relapse from complete remission (CR) with cells still sensitive to the original induction agents (2). The Cancer and Leukemia Group B (CALGB) has completed a pilot study of the feasibility of a pure intensification for complete remitters (32). Patients were induced into CR with mechlorethamine, vinblastine, procarbazine, and prednisone, then, following a rest to allow for complete marrow recovery, were given four cycles of these same agents at augmented dose levels calculated on the basis of tolerance during induction. This regimen was found to be feasible and tolerable, and, while no conclusions regarding efficacy could be drawn from a one-arm pilot, no indication of inferiority to historical experience was evident. For 30 patients in CR who accepted intensification, 92% (actuarial estimate) were relapse-free at $> 1$ year of median follow-up postremission. These data are of value, because they provide sufficient background for prospective trials analyzable for the impact of such intensification on regrowth of biochemically sensitive cells.

The Eastern Cooperative Oncology Group (ECOG) has presented preliminary data concerning patients with advanced Hodgkin's disease who received mechlorethamine, vincristine, procarbazine, prednisone, and bleomycin (MOPP-Bleo) for six cycles. Some patients were randomly crossed-over to three cycles of doxorubicin,

bleomycin, vinblastine, and dacarbazine (ABVD) (33). Almost 60% of > 200 patients achieved CR with MOPP-Bleo, and almost another 25% achieved CR on follow-up treatment with ABVD. At the time of the report, > 90% of patients with CR remained alive at 5 years. For the 60% of the patients induced into CR by the single combination, the use of ABVD during remission is interpretable as a cross-over. This would qualify as an intensification, however, only if maximally tolerable dose levels were employed. For the 40% of patients not achieving remission on MOPP-Bleo, continued use of this regimen could not be considered adequate preparation for a late intensification. Since more than half of these 40% did achieve CR on ABVD, an earlier cross-over might have been preferable. Hence, the use of ABVD following MOPP-Bleo could be the basis for a cross-over intensification, depending on the timing of the cross-over and dose levels of ABVD employed. The efficacy of such a regimen would depend on the biochemical and kinetic composition of the cells residual after MOPP-Bleo. This latter point could be assessed by retreating lymphomas that relapse from the MOPP-Bleo/ABVD regimen with MOPP-Bleo, ABVD, or the combination of both. This would give some idea of the degree of biochemical resistance found in residual populations, and is ethically justified on the basis of retreatment MOPP data (2). It would be particularly instructive to compare the ECOG approach with the use of a strict alternation of MOPP (or its variant) and ABVD as reported from Milan (34). If 60% of patients are induced into CR by MOPP alone, it is possible that these patients would be MOPP-undertreated by diluting 6 months of that regimen over 12 full months of a treatment employing ABVD. A great deal would depend on the overlap sensitivity of populations to MOPP and ABVD. The excellent CR rate reported for the strict alternation (34) is in keeping with preliminary results from other programs (35). However, not all experience with strict alternation has been positive (36). At present, the CALGB is repeating the Milan trial, comparing 12 months of alternating MOPP and ABVD to each regimen alone. This trial and future studies should be analyzed with respect to the implication of various models. Attention should be paid to subsets of patients with clinical characteristics suggesting particular kinetic situations: those with bulk disease, mixed cellularity histology, or marrow involvement would be some examples. It is possible that different subsets of patients may be best served by different approaches.

Two major programs in the therapy of aggressive non-Hodgkin's lymphomas utilized cross-over designs. Both were particularly concerned with diffuse, large-cell histologies. The ProMACE-MOPP regimen first applied prednisone, methotrexate, doxorubicin, cyclophosphamide, and etoposide, in repeated cycles with careful assessment of the rate of tumor volume regression (37). About 40% of patients had a smooth rate of tumor volume regression, achieved CR, and were consolidated by one additional cycle. They were then given the familiar MOPP regimen for a symmetrical duration of time. This is clearly a cross-over at CR, which could be viewed as a new-agent intensification, depending on the dose levels used. Following MOPP, the patients received additional cycles of ProMACE, but now each 2 months to assure that marrow recovery would allow for adequate dose levels of treatment. Another 20% of patients did not achieve CR on their induction exposure to ProMACE, but, on demonstrating a slowing of their initial rate of tumor volume regression, switched to MOPP, which did accomplish CR. These patients received one cycle of MOPP in CR, then additional cycles of ProMACE as a late intensification. Other patients achieved CR on the second application of ProMACE, having demonstrated a slowing of rate of regression on a first application of ProMACE, then a slowing of regression on the MOPP cross-over. These latter patients are an important group because they "failed" ProMACE originally, which is the reason they were switched to MOPP. It is possible that a high percentage of the original tumor was MOPP-sensitive but ProMACE-resistant, so that on the first ProMACE section the dominant MOPP-sensitive cells grew, forcing a switch to MOPP. When the MOPP-sensitive cells were eradicated, however, a residual collection of ProMACE-sensitive, MOPP-resistant cells could be seen, which did enter CR on the reapplication of ProMACE. This is in fact a modeling hypothesis to explain the data, and may be productively pursued in future programs. The total 75% CR rate in this regimen and its durability (a < 25% relapse rate at > 3 years of observation) compare favorably with less complicated regimens, but no prospective evaluation of alternate plans has yet been accomplished. The innovative aspects of this program are the flexibility of the various regimen durations, an attempt at using the rate of tumor volume regression to guide these durations, and the use of intensive therapy after the achievement of CR. This is in marked contrast with a temporarily fixed, relatively low-dose "maintenance" approach to chemotherapy after remission is achieved. ProMACE-MOPP is obviously a treatment plan that could be analyzed from a modeling viewpoint. Several clinical outcomes would be of particular interest in this regard. For example, the relative duration of CR as a function of time to achievement of CR could relate to the relative proportions of ProMACE- and MOPP-sensitive subpopulations within the tumor. Also, the responsiveness of tumors to ProMACE or MOPP at relapse would be useful in assessing the determinants of failure, in particular the relative contributions of biochemical versus kinetic resistance.

The M. D. Anderson program for aggressive lymphomas was not as flexible as ProMACE-MOPP, but it did employ a cross-over as well as a new-agent intensification (38). Patients received three cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP). Those who attained CR received additional cycles, then crossed-over to a regimen which substituted

cytarabine (ARA-C) and bleomycin for cyclophosphamide and doxorubicin. Patients not in CR after 3 months of CHOP also switched to the new regimen, but continued their doxorubicin as well. Late intensification for patients attaining CR by this route was a combination of ifosfamide, methotrexate, and etoposide. The total CR rate on this program was at least 80%, about 80% of these on the initial CHOP. The relapse rate of 4.5 years was < 22%. While not as flexible as ProMACE-MOPP, the use of a fixed time-point for assessment of response may make plans of this type more feasible in a multi-institutional setting. In future studies, carefully chosen control limbs would be needed to assess the relative contributions of new-agent intensification, duration of therapy, or the specific drugs employed.

In the therapy of acute myelogenous leukemia, two forms of postremission treatment may prove interpretable as cross-over intensifications. One of these is the use of ARA-C, alone or with other agents, in one to three high-dose pulses after initial remission induction. This plan is in lieu of low-dose maintenance chemotherapy. Several uncontrolled trials have applied such treatment with encouraging results (39–42). As would be predicted, long delays between the end of induction and postremission intensification have proven detrimental (43–45). An ongoing CALGB study is evaluating the feasibility of intensive ARA-C administration after an ARA-C plus anthracycline induction.

A second form of postremission treatment that has been discussed as a late intensification is the use of marrow-ablative chemotherapy, sometimes with ionizing radiation, followed by transplantation of healthy marrow, usually from a HLA-matched sibling. These nonrandomized trials are difficult to interpret (46), and factors other than cell kill, such as graft-versus-leukemia, may be therapeutic (47). Nevertheless, initial results with marrow-ablative intensification are promising: when compared with chemotherapy-only controls, the relapse-free advantage ranges from 29% to 55% (48–50).

Patients with high-risk acute lymphoblastic leukemia may benefit as much from brief, high-dose postremission chemotherapy as from a more prolonged but less intensive approach (51). In general, the best results are seen in programs employing intensive therapy after remission (52). These observations require evaluation in prospective controlled trials. A modeling analysis may be relevant to a current CALGB trial, exploring an anthracycline intensification after standard induction.

Also of interest is a CALGB trial, recently closed to new patient accrual, in the postsurgical adjuvant chemotherapy of stage II breast cancer. After 8 months of treatment with cyclophosphamide, methotrexate, 5-FU, vincristine, and prednisone (CMFVP), patients are randomized to continue for another 6 months or switch to another doxorubicin-containing combination. This trial does not maximize the dose level of the second combination. Other cross-over trials have been accomplished, but randomized controls are lacking. For example, a complex study in Milan used CMFP postmastectomy at 50% of a "standard" dose level, with escalation over 6 months to triple the initial doses. All patients were then crossed-over to a doxorubicin combination with a similar reduction-escalation scheme. Controls were also crossed-over, but received standard dose levels of chemotherapy throughout (53). No advantage of the reduction-escalation plan was found, but both arms appeared superior to historical experience with non-cross-over programs (54). It is, in fact, remarkable that a 50% reduction in dose from a standard level of chemotherapy can be salvaged by 150% of this standard dose later in the regimen. Both the CALGB and Milan programs demonstrate the feasibility of cross-over designs in the management of early-stage breast cancer and provide background for future studies of cross-over with intensification.

Like Hodgkin's disease, small-cell lung cancer has a high response rate. This disease relapses, however, with great frequency. Aggressive dose levels of therapy are associated with the best clinical results (55), but merely increasing the intensity of induction may not be associated with further improvements (56,57). However, postremission intensification has been associated with some benefit (58). This is in distinction to programs employing strict alternation of different regimens, which have not proven successful (59,60). A small benefit in extensive-stage patients has recently been reported from a chemoradiotherapy induction followed by a cross-over to an alternating program without intensification (61). Other cross-over designs are in progress. These include a CALGB study which uses induction chemotherapy and cranial radiation with or without regional radiation. All patients then cross-over to doxorubicin in combination with some of the original agents. As in leukemia, postremission marrow-ablation trials are also in progress (62).

Several completed trials in the therapy of advanced ovarian carcinomas are of interest. Two effective combinations, HAC (hexamethylmelamine, doxorubicin, and cisplatin) and Hexa-CAF (hexamethylmelamine, cyclophosphamide, methotrexate, and 5-FU), are not more effective when combined in strict alternation (63–65). However, HAC followed by three monthly pulses of moderately high-dose cyclophosphamide may be promising (66). This plan is clearly a cross-over intensification. Also meaningful in this regard is a plan that uses cyclophosphamide, doxorubicin, and cisplatin initially, but discontinues the cisplatin while escalating the dose levels of the other agents after eight cycles (67). The program is completed with several pulses of escalated cyclophosphamide. This design is therefore similar to the ARA-C intensifications in leukemia, in that a component of the induction regimen is given in higher doses as the tumor volume regresses.

## DISCUSSION

This review illustrates that the concept of intensification is clinically feasible, and possibly efficacious. It should be clear that biochemical and kinetic resistance are related topics and that both may be important in hampering our ability to cure cancer. Attention to one by the exclusion of the other may be a serious mistake. It is also clear that few clinical studies have specifically addressed these issues with a randomized, prospective evaluation of alternate schemes of dose and regimen scheduling. Such experiments are clearly needed to advance our knowledge of the complex biology thwarting our therapeutic intentions. Single-arm studies, no matter how seemingly effective (and several employing intensification have indeed seemed effective), cannot provide satisfactory information. It is critical in all of our investigations to retain the concept that a model cannot rise or fall on a single "yes/no" experimental result. This applies not only to the Norton-Simon hypothesis, but all models, the Skipper-Schabel formulation, and the application by Goldie and Coldman of classic mutation theory (68,69) applied to neoplasia (70). We must always examine the appropriateness of the clinical setting and the interpretability of clinical results in terms of the underlying biology. The complexity of clinical oncology indicates that the usefulness of a model cannot be judged based on a single result in a single disease.

This brief review has described the clinical enigma that a decade ago motivated an extension of the Skipper-Schabel model to problems of nonexponential growth. The models are described and the concept of intensification, derived from the models, is presented with clinical illustrations. An enigma to be solved over the next decade is how to properly evaluate these ideas and other ideas derived from this type of mathematical modeling. The solution of this new enigma may be a real step toward improving the application of our growing anticancer pharmacopoeia.

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# The Preclinical Screening Laboratory: Evaluation of Immunomodulatory and Therapeutic Properties of Biological Response Modifiers[1,2]

**James E. Talmadge* and Ronald B. Herberman[3]**

Biological response modifiers (BRMs) are those agents or approaches that modify the relationship between the tumor and host by modifying the host's biological response to the tumor cells, with resultant therapeutic effects (1). BRMs may modify the host responses in several ways:

(*a*) increase the host's antitumor responses through augmentation and/or restoration of effector mechanisms or mediators of the host's defense or decrease the component of the host's reaction which may be deleterious;

(*b*) increase the host's defenses by the administration of natural biologicals (or the synthetic-recombinant derivatives thereof) as effectors or mediators of an antitumor response;

(*c*) augment the sensitivity of the host's tumor cells to endogenous mechanisms for the control of tumor growth;

(*d*) decrease the transformation and/or increase differentiation (maturation) of the tumor cells;

(*e*) increase the ability of the host to tolerate damage by cytotoxic modalities of cancer treatment.

Specifically, BRMs include immunoaugmenting, immunomodulating, and immunorestorative agents; cytokines (soluble protein mediators produced by the host, which include interferons, lymphokines, and monokines); cytokine inducers; inhibitors of tumor growth factors; thymic factors; tumor antigens; and modifiers of tumor antigen cell surface components, antitumor antibodies, antitumor effector cells, and maturation and differentiation factors.

In October 1978, the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), made the decision to organize a program for the study of BRMs. The Board of Scientific Counselors of the DCT established a subcommittee on BRMs, chaired by Dr. Enrico Mihich, to review existing clinical and laboratory experiences with BRMs, to develop guidelines for the development of BRMs as opposed to conventional therapeutic agents, and to provide recommendations for the establishment of a new program. An interim report was submitted to DCT on October 30, 1979, which was subsequently finalized and published (1). In the same month, the Biological Response Modifiers Program (BRMP) was established, following the guidelines suggested by the BRM subcommittee (2). The objectives of the BRMP included the development of a coordinated and focused program of both basic and clinical research on BRMs in the extramural community and the establishment of an intramural laboratory at the NCI-Frederick Cancer Research Facility in Frederick, MD, to perform in-depth laboratory and clinical research on selected aspects of biological response modification and to serve as a source of advice to the Program.

Among the specific recommendations for the BRMP was that a mechanism for preclinical assessment of BRMs be developed along with a BRM Decision Network Committee (DNC). The overall rationale for establishing a Preclinical Screening Laboratory (PSL) and advisory committee for BRMs (DNC) was essentially the same as that for the drug development program for the NCI, ie, the need to evaluate a variety of agents for their therapeutic potential and to select the most promising agents for clinical trials. However, there are some major distinctions between a preclinical screening program for BRMs (3,4) and the previously established screens. The main features of the BRMP's approach to preclinical screening and its major differences from the chemotherapy screens are as follows.

1. The chemotherapeutic screening program has been organized to evaluate large numbers of candidate agents (approximately 10,000 per year), most of which have little or no prior evaluation for antitumor effects. Because of the desire to screen so many compounds, it has been necessary to develop a simple standardized panel of test tumors (5,6) to detect possible antitumor effects at nonlethal doses of the agents. It has become clear that the reliance on screening of potential chemotherapeutic agents

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[3]Preclinical Screening Laboratory, Program Resources, Inc. (*J. E. Talmadge*) and Biological Response Modifiers Program, Division of Cancer Treatment (*R. B. Herberman*), National Cancer Institute-Frederick Cancer Research Facility, MD.

**Reprint requests to:* James E. Talmadge, PhD, Preclinical Screening Laboratory, Program Resources, Inc., NCI-Frederick Cancer Research Facility, PO Box B, Frederick, MD 21701.

on a few transplantable rodent tumors has limited potential for clinical predictions of activity (7-11). At present, other alternatives are being considered, particularly the study of candidate agents in vitro on established human tumor cell lines and in vivo on human tumor xenografts in nude mice. In contrast to the quantitatively extensive but necessarily superficial approach to drug screening, the intent of the BRM screening program has been to develop in-depth evaluation of only a few selected agents (approximately ten to 20 per year), which have been previously assessed in other laboratories and have provided some promising indications for biological response modifying and/or antitumor effects (12).

2. The preclinical screening program for BRMs has been developed to obtain detailed information not only on in vivo antitumor effects but also on an agent's possible mechanisms of action. The essential premise is that an insight into the mechanisms involved in the action of a BRM and the determination of the optimal dose and schedule of administration for affecting biological responses will be important for the comparative assessment of agents and particularly for the rational design of therapeutic protocols.

3. Because of the diverse array of BRMs, no single screening approach is completely satisfactory. For chemical or biological reagents which act on rodent cells, particularly those which have effects on the immune system, a "common-track" screening program has been developed (4,12). However, this approach is not suitable for the evaluation of BRMs with species-restricted activities, eg, some human cytokines, anti-human tumor monoclonal antibodies, and differentiation and maturational factors. For these categories of BRMs, "special-track" screens are now being developed and will include tests against human tumor xenografts in nude mice and in vitro evaluation with human normal and malignant cells and tissues. In 1981, the common-track screen was initiated at the Frederick Cancer Research Facility as the PSL, and this laboratory has been fully operational since 1982.

4. The BRMP's DNC has been established as an advisory committee to assist in the selection of BRMs for screening, to evaluate the result of screening data, and to make recommendations as to the suitability of agents for clinical tests (1,2). It consists of several extramural scientists with clinical and/or laboratory expertise related to BRMs, staff members of the extramural and intramural BRMP, other scientists from the National Institutes of Health, and representatives from the DCT Developmental Therapeutics and Cancer Therapy Evaluation Programs and from the Office of Biologics, Food and Drug Administration. Investigators in academic institutions and biotechnology or pharmaceutical companies who desire the examination of a BRM by the PSL are requested to provide a detailed summary of available information on the compound, describing preclinical and clinical studies of biological and/or therapeutic activities. This information is reviewed by the DNC, and agents which are considered highly promising or representative of a category of BRMs not adequately evaluated for therapeutic potential are recommended for entry into the PSL. Alternatively, additional information and/or further testing is requested from the company, or it is decided that the material is not sufficiently different from an agent previously examined or is not of sufficient interest to the BRMP. Most agents accepted for screening are tested in chronological order, as soon as sufficient endotoxin-free material has been made available. However, agents considered by the DNC to have high priority for evaluation may be tested more expeditiously. Upon completion of the testing for immunomodulatory activity and antitumor effects on experimental and spontaneous metastases, an interim report of the PSL's results is presented to the DNC. Based on the available published literature, information provided by the supplier of the agent, and results obtained in the PSL, the DNC may recommend additional studies, entry into clinical trials, or discontinuation of further involvement with the agent.

5. The BRMP and its DNC have outlined several main objectives for the PSL:

(*a*) the ability to detect the activities of a BRM with well-documented immunomodulatory effects and to observe therapeutic effects with agents previously demonstrated to have clinical efficacy;

(*b*) determination of the predictive value of the PSL's immunomodulation assays for comparable activities in the assays used to monitor cancer patients tested with the same agents;

(*c*) determination of the predictive value of the PSL's results for antitumor activity in cancer patients (to this end, it will be important to clinically evaluate not only agents which were positive in the preclinical screen but also a number of agents that gave negative results in the preclinical screen); and

(*d*) attempt to determine the optimal therapeutic protocol for the administration of a BRM, to provide some guidance for the design of clinical trials with the agent.

In-depth evaluation of even a moderate number of BRMs for their immunomodulatory and therapeutic potential is a formidable task (12,13). The PSL utilizes a series of in vitro and in vivo models which were selected for their expected relevance to the immunotherapy of neoplastic diseases. Each of the assays has been carefully standardized, so that consistent and reproducible effects could be obtained (14). Suitable positive and negative control agents have been included in each assay to determine the technical adequacy of each experiment and to allow comparisons of results on different days.

The PSL uses a sequential and progressively more demanding series of assays designed to determine the range of immunomodulatory effects of a BRM as well as to evaluate its therapeutic potential. A schematic representa-

TABLE 1.—Preclinical screening for BRMs*

| Progression | | | | | |
|---|---|---|---|---|---|
| Activation | Testing | Macrophages | NK cells | T cells | B cells |
| In vitro | In vitro | Culture with BRM for 24-hr, 72-hr cytotoxicity assay of [$^{125}$I] IUdR-labeled tumor cells and/or 18-hr $^{111}$In-labeled tumor cells | Culture with BRM for 24-hr, 4-hr cytotoxicity assay against $^{51}$Cr-labeled YAC | Allogeneic MLR blastogenesis<br>MLTR-CMC allogeneic and/or syngeneic | |
| In vivo | In vitro | Injected BRM iv, harvest AM and test for tumoricidal activity | Inject BRM iv, measure NK activity as above, IFN stimulation | Immunization against TSTA CTL | Stimulation of specific antibody production |
| In vivo | In vivo | | Prevention of metastasis in 3-wk-old mice (fibrosarcoma) | Immunization against TSTA, sc challenge<br>Alteration of tumor growth in UV-irradiated mice after immunization | |
| Mechanism | | Kinetics of activation | Kinetics of activation<br>Induction of hyporesponsiveness | | Duration of response |

* IUdR = iododeoxyuridine; AM = alveolar macrophages; IFN = interferon; MLR = mixed lymphocyte response; MLTR-CMC = mixed lymphocyte tumor response followed by cell-mediated cytotoxicity; TSTA = tumor-specific transplantation antigen; and CTL = cytotoxic T cells.

tion of this approach to the study of defined chemical and biological agents is shown in table 1. The initial emphasis is on defining the effects of potential BRMs on T cells, B cells, NK cells, and macrophages. In addition to the assessment of a BRM's effects on the overall function of the components of the immune system, particular emphasis is placed on determining its ability to augment effector functions which have been shown to have some relevance to host resistance against tumor growth. Thus, evaluation of the induction of tumoricidal activity by T cells, NK cells, and macrophages are major components of the screen. In addition, the adjuvant activities of each agent for T-cell and B-cell responses are assessed. The sequence and progression of assays are as follows:

(*a*) In vitro activation followed by in vitro testing
(*b*) In vivo activation followed by in vitro testing
(*c*) In vivo activation followed by in vivo testing.

This sequence of studies allows the determination of the effective dose range and any possible concomitant toxicity. Information is also obtained about the kinetics and duration of the effects of an agent, which can guide the planning of therapeutic protocols. There are occasional BRMs which cannot be adequately tested in certain assays, such as those agents which are not soluble in medium or which require in vivo activation and thus cannot be examined within the in vitro activation, in vitro testing procedures. On the other hand, adequate evaluation of some agents may require additional assays of relevant or expected activities, and attempts are made when necessary to add such tests to the standard assay procedures. The initial assays are performed with cell populations obtained from normal mice and from normal mice treated with a BRM, in order to define the range of biological activities in the absence of tumor-induced changes in host reactivity, eg, tumor-induced immune depression or tumor-associated suppressor cells. Additional studies are undertaken to determine the nonspecific immunoprophylactic activity of a BRM, ie, the ability of pretreatment with a BRM to protect against the development of metastases following iv tumor challenge. Studies are also undertaken to examine the ability of a BRM as an adjuvant to induce a tumor-specific immunoprophylaxis in conjunction with a tumor vaccine, resulting in the rejection of a tumor challenge in syngeneic mice. We also examine the ability of a immunomodulator to act as an adjuvant for a tumor vaccine in animals which contain UV-induced suppressor T cells (15).

Because metastasis is a major threat to cancer patients, the emphasis in the therapeutic evaluation of BRMs is on the assessment of therapeutic efficacy against systemic lesions (table 2). Two models of immunotherapy are utilized, one directed against tumor cells introduced in the

TABLE 2.—Preclinical screening of BRMs for immunotherapeutic effects

| Treatment of metastases | Treatment of autochthonous tumors |
|---|---|
| 1. Experimental metastases | 1. UV-induced skin tumors.<br>BRM given: |
| 2. Spontaneous metastases<br>Efficacy indicated by increase in survival and/or decrease in number of metastases | a. iv, administered long-term<br>b. intralesionally |
| | 2. NMU-induced mammary tumors in rats.<br>BRM given:<br>a. iv, administered long-term<br>Efficacy indicated by increase in survival, reduction in growth rate, and decrease in metastases |

host systemically by iv injection (experimental metastasis) and the other, more clinically relevant assessment of activity against metastatic foci which develop after the resection of a primary tumor (spontaneous metastasis). In the spontaneous metastasis model, the host has been "conditioned" by tumor growth for some period of time prior to initiation of therapy following surgical resection of the primary tumor. This is in contrast to therapeutic models directed against metastatic disease, in which therapy is initiated 1 or 2 days following primary tumor transplantation and represents a form of prophylaxis, since therapy is started prior to the tumor cell dissemination to form metastases. Furthermore, with the latter type of model, the primary tumor burden in the treated animals is often smaller than in saline-treated hosts, thereby reducing metastatic disease and preventing a valid comparison of metastatic burden with the control animals which have a more extensive primary and potential secondary tumor burden. Therefore, it is expected that the post-resection experimental design will be more predictive of systemic therapeutic efficacy in the clinical setting (13), while the latter tumor model might give an overly optimistic assessment of the efficacy of an agent for the treatment of pre-existing micrometastases.

It is likely that, as one progresses from models of immunoprophylaxis to more stringent and rigorous models of immunotherapy, fewer positive results will be obtained. Thus, a BRM with prophylactic activity may not have striking therapeutic activity against established systemic disease in a relatively normal host (experimental metastasis), or in a host conditioned by the growth of primary tumor, with pre-existent spontaneous metastases. However, even the spontaneous metastasis model utilizes young, relatively healthy hosts bearing transplantable tumors, a situation which may be quite disparate from that of cancer patients who may have been exposed to carcinogenic insult many years before and who have gradually developed progressive primary and metastatic tumor lesions over a very prolonged period. Thus, one might expect that the most clinically relevant model would be primary, autochthonous tumors that metastasize.

To this end, we have developed two metastatic autochthonous tumor models (13). The first utilizes UV light-induced skin tumors, which include a variety of histologic types: squamous cell carcinomas, fibrosarcomas, hemangiosarcomas, rhabdomyosarcomas, and other less well differentiated sarcomas. The tumors develop 20-50 weeks following the onset of UV irradiation (2.8 Joules/$m^2$/minute for 2 hours, three times/week, for 8 months). The second model utilizes the induction of mammary tumors in Sprague-Dawley rats by *N*-nitrosomethyl urea (NMU). The NMU is injected iv into 50-day-old female rats at 35 mg/kg. This dose provides an acceptable balance between the tumor induction time (90-120 days) and the induction of a limited number of primary tumors (13). Higher doses of NMU result in the more rapid development of an initial lesion in 100% of animals but also result in multiple primary neoplasms, such that few animals survive to form apparent metastases (13). In both therapeutic models, when palpable tumors are detected the animals are randomly assigned to one of several arms of a protocol and treatment is initiated. The therapeutic efficacy is determined based on the survival of animals, rate of tumor growth, and development of metastatic lesions and multiple primary tumors. These studies are more difficult, tedious, and time-consuming than experiments with transplantable tumors but provide a rigorous and hopefully more predictive test of therapeutic efficacy. The difficulties associated with such studies are similar to those routinely encountered in clinical trials, including: variations and delays in tumor accrual, need for randomization between treatment and control groups, variability of tumor growth and behavior, need for extensive and relevant statistical evaluation, and prolonged study duration.

Obviously, a balance has to be reached that provides an acceptable compromise between the requirement for clinically predictive therapy models and the fiscal and time constraints that bias one towards the use of experimental or spontaneous tumor metastasis models. To achieve the parallel goals of predictive and realistic therapy models, our initial studies to determine optimal doses and schedules of administration are performed with the experimental and spontaneous metastasis models. Based on the results of these studies, we continue with more limited studies utilizing autochthonous tumor models (table 2). Thus, the initial assessment of therapeutic activity using a broad range of doses and therapeutic schedules can be undertaken for the treatment of experimental metastases, and data are obtained within 6-8 weeks. Selected schedules and doses (three to five doses, one to three schedules) are then examined in the therapeutic models utilizing spontaneous metastases, and data are obtained in 12-16 weeks. Then, using the optimized schedule and one to three doses of the BRM, we examine the therapeutic activities of individual BRMs in the treatment of autochthonous tumors, and survival data are obtained approximately 1 year following the initial development of a palpable tumor. It has become apparent from these studies that the use of autochthonous UV-induced tumors and the NMU-induced mammary tumors provides a logistical and technical challenge to the evaluation of the therapeutic potential of a BRM. However, because of the considerably closer analogy of the autochthonous tumor models to the clinical situation, we anticipate that positive therapeutic efficacy in the autochthonous tumor models will be more predictive of clinical efficacy than the simpler and less demanding metastasis models. In the development of the therapy models for the PSL, it was apparent that no one animal tumor model is optimal and that all suffer from various limitations and discrepancies from the clinical situation. The adequacy of one tumor as compared to another to assess the therapeutic efficacy of

a BRM is of secondary importance compared with the rational development and interpretation of results from the model system. Thus, the aim has been to develop reproducible, standardized, and interpretable models of immunotherapy which may have some predictive value.

The results from the preliminary studies with a number of agents (table 3) demonstrate that such a systematic preclinical approach is able to delineate various classes of effector cell activity. Table 3 presents some of the observations from the PSL utilizing representative immunomodulators and demonstrates that a variety of compounds may have different activities in vitro as opposed to in vivo. As an example, MVE-2 is unable to augment NK cell activity in vitro but is capable of augmenting NK activity in vivo, presumably secondary to interferon stimulation (16). In contrast, other compounds such as Nor-MDP and recombinant IL-2 have far less activity in vivo due to their very short serum half-life, which can be partially overcome if high levels of these immunomodulators are administered in vivo (17). Adjuvant-like activity is found with a number of different classes of immunomodulators, including poly ICLC and MVE-2, which are macrophage-activating agents but have no apparent direct effect on T cells (16–19). In contrast, an agent such as thymosin fraction 5, which has no macrophage-augmenting activity, also has potent immunoadjuvant activity (20,21). Thus, it appears that various classes of immunomodulators can have adjuvant-like activity, due to either T-cell effects or macrophage-augmenting effects.

The comparison of preclinical data from immunomodulatory analyses with therapeutic properties and/or prophylactic properties of various immunomodulators can be very informative (table 4). It should be noted (*see* table 3) that most of the agents examined to date in the PSL have usually had multiple immunomodulatory effects, ie, they have activated or augmented several different effector cell activities. In addition, a considerable proportion of the agents tested (presumably due to preselection) had significant antitumor activity and, as might have been anticipated, the nonspecific prophylaxis model gave more positive results than the therapeutic models. The comparisons between the immunomodulatory and the prophylactic properties suggested that nonspecific immunoprophylaxis was associated with NK cell-augmenting agents. This was supported by experiments utilizing anti-asialo GM1, which was found to ablate the prophylactic-like activity of the agents (22,23). The correlation of immunological effects with therapeutic activity was not so clear; however, there was a trend towards a correlation of therapeutic efficacy with macrophage activation and in the case of thymosin fraction 5, with T cell-stimulating agents, but there was no correlation with systemic NK-augmenting effects.

Over the short span of the existence of the PSL, there have been major changes in the types of BRMs available for screening. Many of the recommendations of the original BRM subcommittee focused on the use of chemicals and crude extracts and the use of interferon as a natural, partially purified material obtained by stimulation of the appropriate cell types. At the initiation of the Program, it was anticipated that interferon and perhaps one or two other recombinant biological compounds might be cloned and evaluated for clinical use within the following 5 years. However, the accelerating technology for the characterization and cloning of cytokines and the manufacture of recombinant molecules have resulted in considerably more genetically engineered BRMs than was initially predicted by experts in the field. There are now several families of biologicals (alpha, beta, and gamma interferon; interleukin's 1, 2, and 3; tumor necrosis factor; lymphotoxin; etc) where one or more recombinant molecules are, or soon will be, available.

Our experiences to date with the preclinical screening of BRMs have provided many new insights into the problems and complexities of such an approach. Although many of these lessons may appear obvious or trivial, they have a large impact on the day-to-day operation of a reproducible screen. One of the most important issues is the need to incorporate both positive and negative con-

TABLE 3.—Comparison of immunomodulatory properties*

| | MVE-2 | Poly ICLC | A/D Bgl | OK-432 | Lentinan | Nor-MDP | MTP-PE in MLV | Thymosin F5 | NED-137 | IL-2 |
|---|---|---|---|---|---|---|---|---|---|---|
| In vitro | | | | | | | | | | |
| T-cell functions | 0 | 0 | – | + | 0 | + | 0 | ++ | 0 | + |
| NK activity | + | + | + | + | 0 | 0 | 0 | 0 | 0 | + |
| Macrophage-mediated cytotoxicity | 0 | + | + | + | 0 | + | + | 0 | 0 | + |
| In vivo | | | | | | | | | | |
| T-cell functions | + | + | + | + | + | ++ | 0 | ++ | + | ++ or –† |
| NK activity | + | + | + | + | + | 0 | +‡ | 0 | 0 | + |
| Macrophage-mediated cytotoxicity | +/0 | ++ | + | + | 0 | + | + | 0 | 0 | + |

*0 = addition of BRM had no effect on effector cell function; – = addition of BRM depressed effector cell function; and + = addition of BRM stimulated effector cell function.

†High IL-2 levels depressed cytotoxic T-cell activity, while low doses stimulated such activity.

‡NK activity noted only in lungs and liver but not in spleen, peritoneal cavity, or blood.

TABLE 4.—Correlation of effector cell augmentation and therapeutic activity*

| Agent | NK activation (spleen) | Macrophage activation | T-cell stimulator in vitro | T-cell adjuvant activity | Nonspecific immunoprophylaxis | Therapeutic activity |
|---|---|---|---|---|---|---|
| MVE-2 | + | +/0 | 0 | + | + | 0 |
| Poly ICLC | ++ | ++ | 0 | ++ | + | ++ |
| A/D BgL IFN | + | + | – | + | + | + |
| OK-432 | ++ | + | + | + | + | + |
| Lentinan | + | 0 | 0 | + | + | 0 |
| Nor-MDP | 0 | + | + | ++ | 0 | + |
| MTP-PE in MLV | 0† | ++ | + | ++ | + | ++ |
| Thymosin F5 | 0 | 0 | ++ | ++ | 0 | + |
| NED-137 | 0 | 0 | 0 | + | 0 | 0 |
| IL-2 | + | + | ++ | ++ or -‡ | + | ++ |

* + = addition of BRM stimulated effector cell function; +/0 = slight stimulation of effector cell function; 0 = addition of BRM had no effect on effector cell function; and - = addition of BRM depressed effector cell function.

† NK cell activity noted in the lungs and liver but not in the spleen, blood, or peritoneal cavity.

‡ High IL-2 levels depressed cytotoxic T-cell activity, while low doses stimulated such activity.

trols into every assay and therapeutic study. This does not eliminate problems, but it does identify intrinsic assay variation and those assays that are technical failures. In addition, positive controls provide a relative assessment of the quantitative ability of the test BRM to affect the assay relative to the potency of the positive control.

One example of the importance of a positive control can be found in therapeutic studies. If one waits too long following the iv injection of tumor cells or if one amputates the primary tumor at too large a size, the tumor burden may be too extensive for immunomodulation to have significant therapeutic activity. Under these circumstances, a positive control as well as a potentially positive BRM under evaluation may have no activity due to the overwhelming tumor burden. Thus, in a study in which the positive control had no therapeutic activity, the validity of the therapeutic assay is questioned and the experiment is repeated before any conclusion is reached regarding therapeutic efficacy of the test agent. Alternatively, if vigilance is not maintained for endotoxin contamination, the test agent may give spurious therapeutic activity.

A positive control can assume several different forms, depending on the assay and effector cells under study. Thus, polyinosinic:polycytidylic acid (poly I:C) provides a useful positive control for NK-cell and macrophage activation but is of little use in T-cell assays. Conversely, the ability of a particular BRM to stimulate a specific effector activity does not imply that it will not concomitantly stimulate other effector activities (eg, interferon activates not only NK cells but also macrophages). A common source of error is the augmentation of NK cells, which can contribute to the cytotoxic activity observed in assays of cytotoxicity by T cells or macrophages.

In assays of specific cytotoxic T effector cells, the sensitivity of the target cells to damage mediated by NK cells or macrophages must be considered. A target cell line that is sensitive to NK cell cytotoxicity may exhibit a sufficiently high background lysis to obscure any superimposed specific T cell-mediated cytotoxicity. Another problem that can be avoided is the use of a tumor line that produces a cytokine which could stimulate effector cell activity. Examples of this problem are the production of IL-2 and other lymphokines by EL-4 (24,25), or IL-1 by P388-D1 (26).

An additional consideration regarding the target cell line includes the observation that the in vitro passage of tumor cells can influence their biological characteristics (27-29). Tumor cells which are established in culture represent only those cells within the primary tumor that are capable of in vitro growth, which introduces the problem of selection. Moreover, in vitro culture of tumor cells may also modify their antigenic characteristics (30-32) and NK susceptibility (33,34), resulting in an additional inadvertent selection of cellular subpopulations. The in vitro culture of tumor lines has been utilized as a technique to develop variants which are sensitive to NK activity and thus have depressed metastatic properties (35). Furthermore, the malignant properties expressed following the injection of cell lines propagated either in vitro or in vivo will be disparate (36). For example, we have found that recipients survive about the same length of time (approximately 30 days) following the ip injection of 5000 ascites-passaged MBL-2 tumor cells or the ip injection of 100,000 tissue culture-propagated MBL-2 tumor cells.

The techniques used to transplant tumors are also critical and influence the composition of a tumor population. Because of this, the tumor used in one laboratory may not have the same biological characteristics as a presumably identical tumor grown in another laboratory. Most commonly, tumors are serially transplanted by trocar passage of tumor fragments. To passage a tumor, a mouse bearing the "best tumor" (eg, nonnecrotic) is selected, and fragments from this tumor are passaged to a second group of mice. This repeated passage of tumor fragments from fast-growing neoplasms may lead to the limitation or abrogation of phenotypic heterogeneity (37,38). To maintain the biological diversity of a tumor,

serial transfers should be done with cell suspensions obtained either by physical dissociation or, preferably, by enzymatic digestion.

The use of tumor cells infected with micro-organisms as target cells in immunologic assays or as inocula into animals has ramifications beyond in vitro cytopathogenic effects or host infection. Tumor cells that are persistently infected with a virus or *Mycoplasma* may not be tumorigenic (39–41). Moreover, the tumors that do develop may not metastasize. The rejection of virally infected cell lines is mediated by an active host process and may be abrogated by prior irradiation of the host or pretreatment of the host with anti-interferon globulin or antilymphocyte serum. In addition, tumor cells infected with viruses often have increased susceptibility to killing by NK cells (42–44). Furthermore, a cell line that is infected with *Mycoplasma* will incorporate higher levels of [$^{125}$I] iododeoxyuridine ([$^{125}$I]IUdR) than the same cell line which is not infected by mycoplasma; this increased incorporation of [$^{125}$I]IUdR can result in radiochemotoxicity to the cell line, thereby affecting the subsequent use of such a cell line in assays such as those of macrophage-mediated cytotoxicity or analyses of tumor localization, survival, and clearance.

Viruses that infect rodent colonies are nearly ubiquitous and perturb immunological studies (45–53). Acute or early infections can be immunostimulatory, whereas chronic infections are generally immunosuppressive. The viral infections which induce immunological disturbances include most common murine viruses. It serves little purpose to investigate the activity of a BRM in an infected animal, since the data cannot be interpreted. Thus, only those animals and tumors that are free of pathogenic murine viruses should be used in the screening of BRMs. During an outbreak of murine hepatitis virus in our animal colony, we were unable to undertake any NK-cell or macrophage analyses, since on entry into the colony the mice were infected and developed maximum levels of effector cell activity. In addition, the high NK activity levels had a significant impact on the development of both spontaneous and experimental metastases and would also have affected any therapeutic analyses that might have been undertaken. Unfortunately, the only practical approach to such a problem is to kill all the animals and decontaminate the vivarium and any additional areas that might also harbor residual virus.

Lack of uniformity in animal husbandry can bring about discrepancies in experimental results. Subtle and unsuspected factors may influence the outcome of otherwise well planned experiments. These can include hyperchlorination of drinking water (54) or the addition of the anticaking agent silica dioxide to autoclavable feed, both of which are common husbandry techniques that depress the number and function of macrophages in rodents. The macrophages of mice housed in over-crowded and dirty cages may become activated to become cytotoxic to tumor cells in vitro (55). Moreover, if adult male mice are removed from their cohort group and mixed with males of other groups, they fight, resulting in macrophage activation presumably due to the systemic induction of endotoxin following skin infection. Thus, a seemingly innocuous experimental manipulation such as mixing cohort groups of donor male mice easily influences the immune status of the test animals. Similarly, NK cell activity is also perturbed by housing and feed conditions (56).

The investigation of a putative BRM needs to be studied within suboptimal conditions (20). For example, in studies dealing with the ability of a BRM to act as an adjuvant in a vaccine to prevent the growth of a tumor challenge, a highly immunogenic tumor used as a vaccine would lead to the rejection of a tumor challenge irrespective of any adjuvant-like activity associated with the BRM. Therefore, immunization protocols, in addition to other immune assays, need to be suboptimal, inducing only a minimal immune response that is incapable of rejecting the tumor challenge unless the vaccine incorporates an effective adjuvant.

One of the major problems encountered by investigators in tumor immunology is the frequent contamination of materials and biologicals with endotoxin. Indeed, it is impossible to properly perform NK cell assays, T-cell assays (MLR and MLTR-CMC), and macrophage assays in the presence of unknown or minimal levels of endotoxin. Perhaps the most sensitive assay is the macrophage migration-inhibition factor (MIF) assay, which is sensitive to picogram levels of endotoxin (57–59), while the macrophage-mediated cytotoxicity assays are sensitive to approximately 1–5 ng/ml of endotoxin (60–62). Such observations demonstrate the exquisite sensitivity of immunologic assays to endotoxin and emphasize the requirement for rigorous testing of endotoxin contamination. Irrespective of the care one takes to prevent endotoxin contamination, it is mandatory that all materials be screened for endotoxin contamination by the *Limulus* lysate assay just prior to their incorporation into an assay. If materials are found to be contaminated, the biological activity of lipopolysaccharide (LPS) can sometimes be neutralized with polymyxin B (60). This antibiotic irreversibly binds to the lipid A moiety of LPS, which is the biologically active portion of the LPS (61). However, since polymyxin B might also influence an immune assay, the best laboratory practice is to avoid working with reagents contaminated with LPS.

The difficulties experienced in immune assays and cell cultures which are attributable to lot-to-lot variation in serum characteristics are well documented. In addition to the utilization of a serum lot which promotes cell division and does not inhibit effector cells, consideration must be given to the type of serum used within each assay. While fetal bovine serum (FBS) or another bovine source of serum is traditionally used for in vitro assays, other serum sources, including homologous serum in rodent studies, should be considered (20). In the in vitro studies of T-cell function (MLR and MLTR-CMC) we have found

that a half percent normal mouse serum is far superior to FBS in the medium (20). Inarguably, it is possible to prescreen serum lots and obtain a useful lot of FBS; however, this may require extensive screening, and variation occurs whenever a new lot must be obtained. Serum choice is also important in studies on the induction of cytotoxic T cells (CTLs) following tumor vaccination. CTLs need not be directed against tumor-associated antigens but rather may be directed against the antigenic FBS proteins, resulting in a loss of specificity of the induced CTLs or in the generalized resistance to any syngeneic tumor challenge with tissue culture-propagated cells (30–32).

Several technical considerations must be addressed when choosing a tumor model to study the activity of a BRM. One important consideration is the use of either a suspension cell culture or an adherent cell line. The use of an adherent tumor line requires trypsinization for harvesting and an incubation period (> 4 hours) to allow for the recovery of antigen expression that may have been affected by the enzymatic treatment. The kinetics of tumor cytolysis (as assayed by radioisotope release) by effector cells also differ between tumor cell lines of lymphoid origin or adherent target cell lines of parenchymal origin. This is very important in CTL assays and macrophage assays. We have found the 72-hour assay for macrophage cytolysis to be much more sensitive than an hour release assay with lymphoma cell lines (18). Indeed, even when adherent targets are utilized in an Indium ($^{111}$In)-release assay there is a requirement for a 48-hour incubation rather than an 18-hour incubation. Tumor targets that are rapidly lysed, such as those of lymphoid origin, are generally assayed using a 4–6-hour radiorelease assay with $^{51}$Cr as the radiolabel. However, the rapid spontaneous release associated with adherent cell lines radiolabeled with $^{51}$Cr precludes their use when longer assays, eg, 16–24 hours or longer, are needed to demonstrate cytolysis. The use of adherent targets requires other radioisotopes such as [$^{125}$I]IUdR, [$^{75}$Se]methionine, [$^{3}$H]thymidine, $^{111}$In, [$^{3}$H]proline, or [$^{3}$H]leucine.

Since many, if not all, BRMs have pleiotropic activities, it is desirable to thoroughly assess the effects of a BRM on each of the main components of the immune system. This contrasts with traditional approaches to the testing of an agent, in which only a limited array of effector cells or immune functions are examined. One example of an unanticipated observation derived from our broad and systematic preclinical screening has been the observation of recombinant IL-2-induced activation of tumoricidal macrophages. Based on current concepts of immunomodulation by IL-2, one would not have anticipated such a result, and it was obtained only by the use of the systematic screening approach.

Although the routine analyses outlined in tables 1 and 2 are very broad and involve the analysis of most major effector cell functions that may have therapeutic activity against tumors, it has not been unusual for additional questions to arise or for other, more specific, analyses to be required for the adequate assessment of some agents, several of which are listed in table 5. Because most of the agents which entered the PSL had effects in multiple assays, this raised questions as to whether multiple cell types were being activated and whether some of the assays designed to measure one cell type might also have been measuring the activity of an unrelated cell type. Therefore, we often undertake studies to determine the nature of the activated effector cell, which may include depletion studies with anti-asialo GM1 serum (to remove NK cells) or anti-Thy 1 serum (to remove T cells) plus complement. However, because anti-Thy 1 will also decrease NK cell activity, even this technique is not definitive. Therefore, additional studies may need to be undertaken, which can include in vivo depletion studies with anti-asialo GM1 (for NK cell depletion); neonatal thymectomy, x-irradiation, and bone marrow reconstitution (for selective T-cell depletion); utilization of nude mice (T cell-depleted) and hyperchlorination of drinking water (to inhibit macrophage function); or use of indomethacin (to depress prostaglandin levels). In addition, in vitro manipulations prior to effector cell analyses may involve specific effector cell depletion or purification of specific subclasses or classes of effector cells.

It has also been helpful to analyze the induction of suppressor or helper cells by a BRM if the routine screening results suggested their presence. As a test of helper/suppressor cell activity, various numbers of cells from a primary MLTR culture may be added to normal spleen cells in a second MLTR assay, to determine their influence on the development of an effector cell population. Alternatively, cells from a vaccinated animal or a primary MLTR culture may be added as putative helper/suppressor cells to an appropriate MLR assay. The Lyt phenotype of putative suppressor cells, helper cells, or effector cells may also be determined with the appropriate antisera.

Individual BRMs can have different kinetics of activation or compartments of activation, predicated on the route of administration. For example, MVE-2 or liposomes incorporating MTP-PE will activate peritoneal macrophages but not alveolar macrophages following ip injection (16). Conversely, alveolar macrophages are activated following iv injection, while peritoneal macrophages are not. There is an increasing awareness within the PSL, based on the differences in activation of various effector cells at particular sites, that it is often necessary to examine effector cell activity in various tissues (particularly those commonly affected by metastases, such as the lungs and liver), as well as within the tumors themselves. For example, it has been reported that multiple injections of interferon result in an NK cell hyporesponsive state within the peripheral blood lymphocytes of patients (63,64) or the spleen cells of mice (65). Similarly, preclinical studies with agents such as MVE-2 revealed an NK cell hyporesponsive state within the spleen, whereas

TABLE 5.—Nonroutine analyses useful in the study of BRMs

1. Characterization of in vitro effector cell populations by:
   a. Depletion
   b. Antiserum sensitivity
   c. Purification of effector cells
2. Examination of synergistic actions of BRMs
   a. Combination chemoimmunotherapy
   b. Combination of BRMs
3. Direct cytotoxic activity
4. Stimulation of cytokine production
5. Immunomodulation in tumor-bearing animals
6. Induction of helper or suppressor cells
7. Compartmentalization, cell number, and differential of induced effector cells
8. Therapy parameters: dose, route, schedule, duration
9. Pharmacokinetics and biodistribution
10. Toxicity

there was a marked increase in the number and function of NK cells in the lungs and liver (16,18,22,23,66). This site specificity may be attributable to in situ proliferation of effector cells in these organs and/or sequestration of NK cells from the circulation. Another example is the observation that liposomes incorporating MTP-PE are unable to augment NK cell activity either in vitro or in the peritoneal cavity or spleen following ip or iv injection, respectively (66); however, NK activity was augmented in the lungs and liver following iv injection. Studies such as these demonstrate the need to examine various tissues for effector activity.

The importance of the route of administration goes beyond its influence on the site of activation. It also impacts on the bioavailability and serum half-life of the immunomodulator. This is readily apparent when one undertakes the examination of the pharmacokinetics and biodistribution of an immunomodulator. For example, following iv injection, recombinant IL-2 has a very short serum half-life and requires very high doses to augment NK cell activity. In contrast, the ip injection of recombinant IL-2 results in a prolonged serum half-life, with concomitant augmentation of peritoneal NK cells at much lower doses of IL-2 than those required to augment splenic NK cells following iv administration (17,67).

The initial immunomodulatory studies in the preclinical screen are based on the effects of a single dose of a BRM. However, therapeutic protocols with BRMs or chemotherapeutic agents rarely utilize a single dose. Thus, the effects of a BRM on immunologic functions, following long-term administration, need to be examined with the goal of determining schedules of administration which continuously or repeatedly modulate effector cell functions.

The development of successful immunotherapeutic protocols requires new strategies as well as the development of clinically predictive tumor models and protocols (13). Beyond the determination of a maximum tolerated dose (MTD), it is also important to determine the optimal immunomodulatory dose (OID) not only in animal models but also clinically. However, in a variety of situations, it should be determined whether either the MTD or OID represents the optimal therapeutic dose (OTD). The OTD appears to be dependent not only on the augmentation of effector cell activity but also on the number, function, and tumor infiltration of effector cells. Furthermore, the determination of an optimal therapeutic protocol (OTP) requires additional information on therapeutic schedule and duration of treatment. Studies of scheduling parameters have revealed that many BRMs induced host toxicity when delivered frequently at high doses. However, if the BRM is administered at either a lower level or less frequently there may be a decrease in toxicity which may be paralleled by greater therapeutic activity. Thus, in agreement with studies of in vitro and in vivo immunomodulation, it has not been unusual to observe a bell-shaped dose-response curve for therapeutic activity. This may be due to decreased immunomodulatory potential at higher BRM doses or it may be associated with toxicity to the host at the higher levels of the agent, which in combination with the tumor burden may result in morbidity or decreased survival as compared to an optimal therapeutic protocol.

In our initial therapeutic studies, we deliver the BRM twice a week for 4 weeks. This schedule is utilized with at least five different doses of the BRM, and if no therapeutic or toxic activity is observed, additional studies are undertaken until an MTD is determined. This second study might also include the daily administration of the agent, in addition to the biweekly administration, to achieve an MTD level. If no therapeutic activity is observed with either type of schedule, we conclude that the agent may have no therapeutic activity on the basis of either immunomodulation or direct chemotherapeutic activity. It should be noted, however, that therapeutic activity may not be obtained in all standard models of immunotherapy. For example, some BRMs, eg, lentinan, have had therapeutic activity only in specific strains of mice. Lentinan has had no detectable therapeutic activity against B16-BL6 in C57BL/6 mice but has been active against the Madison 109 tumor in BALB/c mice.

If therapeutic activity is observed in the initial dosage studies, additional studies are undertaken with a limited number of active doses. The test BRM at one to three doses is administered once a week, twice a week, three times a week, and daily for a 4-week period. This second-phase study for the determination of an OTP utilizes an experimental design such that the daily administration is biased towards lower, more easily tolerated doses, whereas a broader range of doses are tested by weekly or biweekly administration. A third phase of studies is designed to determine the duration of administration required for optimal therapeutic activity. Thus, studies may be undertaken in which the agent is delivered by an

optimal schedule for 1-4 weeks, or continuously as long as the animals remain alive. In studies with the autochthonous tumor models, particularly the NMU-induced tumor model in which animals often develop multiple primary tumors, we have found that maximum therapeutic activity, as determined by long-term survival, is dependent upon continuous administration of the BRM. Thus, we suggest that the demonstration of clinical immunotherapeutic activity may also require the prolonged administration of a BRM.

The OTP appears, at present, to be dependent on a complex combination of parameters including dose, frequency of administration, duration of administration and route of administration. The route of injection has been shown to be important, in that ascites tumors have responded most favorably to the ip administration of an agent, whereas systemic disease following iv injection of tumor cells or spontaneous metastasis may be less responsive to ip injection of an agent, which, as discussed earlier, may result in only a regional augmentation of effector cell activity. Normally, systemic disease is treated by iv injection; however, consideration must also be given to im administration, which may be beneficial because of a prolonged half-life of the agent in addition to the more convenient injection site. Indeed, in our studies with long-term administration, particularly in animals receiving daily injections of a BRM, it becomes impossible to continue the iv lateral tail vein injection and one must resort to alternative injection sites. For agents which can be absorbed through the gastrointestinal tract, oral administration also has considerable clinical practicality, since it may allow ongoing treatment on an outpatient basis. Thus, administration of BRMs via gavage should also be considered for immunotherapeutic studies.

In conclusion, the PSL has demonstrated that it is possible to efficiently generate a body of reproducible data on a series of widely disparate BRMs, which can then be examined for possible correlations, retrospective or prospective, with clinical immunomodulatory and therapeutic effects. Our initial examination of possible clinical correlations has pointed toward the need for future clinical trials to include an assessment of immunological parameters as similar as possible to those used in the preclinical screen, including not only differential counts of circulating leukocytes and NK cell augmentation studies but also monitoring of MLRs, monocyte-mediated cytotoxicity, and the ability of T cells to develop specific cytotoxic reactivity in vitro. Conversely, it may be useful to incorporate into the preclinical studies the phenotypic analysis of subpopulations of spleen cells from animals receiving long-term BRM administration, to provide analogous data to those obtained by cytofluorometric analyses in clinical trials. The determination of the actual value of the PSL will come from experience over the next several years as agents evaluated in the screen are tested clinically. We hope to determine how useful the screen results are for help in designing clinical treatment schedules, determining appropriate monitoring assays, and predicting therapeutic efficacy.

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# Pathogenesis of Metastatic Disease: Implications for Current Therapy and for the Development of New Therapeutic Strategies

George Poste[1,*]

**Different tumor cell subpopulations coexisting within the same tumor exhibit varied susceptibilities to antineoplastic agents. Tumor cell heterogeneity is now recognized as the principal cause of treatment failure in cancer, and is a formidable obstacle to effective therapy and to the development of drug delivery systems for selective targeting of antineoplastic agents to tumor cells. Recent insights into the genesis of tumor cell heterogeneity during progressive tumor growth reveal new complexities that raise challenging questions about the adequacy of certain approaches to the current therapy of metastatic disease and impose challenging criteria for the development of improved therapeutic strategies. Many of the experimental approaches used in the search for new antineoplastic agents and targeted drug delivery systems ignore the pathogenesis of metastasis and the problem of tumor cell heterogeneity. The adoption of more relevant assay systems is an urgent priority. These include the greater use of metastatic tumor models and the increased use of human tumor cells to replace rodent cell systems which have been of limited predictive value in identifying effective anticancer agents. In contrast to current strategies for the development of new antineoplastic drugs which seek to identify agents with activity against a broad range of histologically diverse tumors, greater success may be achieved by seeking agents active only against specific cell lineages. Many established human tumor cell lines may not be suitable for this purpose because of extensive phenotypic change produced by prolonged passage ex vivo. Development of histiotype-specific human tumor cell screens will require an extensive research effort to identify target cells that display demonstrable phenotypic relatedness to tumor cells in neoplastic lesions. Major advances in the therapy of metastatic disease are considered unlikely in the next few years, and progress will stem from improved use of existing agents in refined combination therapy protocols in which greater attention is given to the duration, frequency, and sequence of therapy with different agents to limit emergence of tumor cell variants resistant to one or more antineoplastic agents. Advances in molecular biology offer exciting prospects for the identification of new therapeutic targets in human tumor cells, for the induction of alterations in tumor cells that could serve as therapeutic targets, and for the elucidation of the mechanisms responsible for the rapid phenotypic diversification of tumor cells. These opportunities will not be realized, however, unless more stringent experimental systems are adopted and refined at regular intervals to accommodate new knowledge about the cell biology of metastasis. [Cancer Treat Rep 70:183–199, 1986]**

Cancer therapy, and chemotherapy in particular, is entering a critical period. Notwithstanding the gains made in the last 20 years in the therapy of lymphoproliferative neoplasms and a few solid tumors such as choriocarcinoma, Wilms' tumor, and testicular cancer, the treatment of most solid malignant tumors and their metastases remains depressingly unsuccessful. The more common human malignancies arising in breast, lung, colon, and the prostate continue to resist therapy in the majority of patients, despite assault by ever larger combinations of drugs. The merits of current approaches to cancer chemotherapy are being challenged on several fronts. The first stems from concern regarding whether the adverse impact of many therapeutic regimens on patient quality of life is justified by the results obtained. The second concern is more fundamen-

[1]SmithKline and French Laboratories, Philadelphia, PA, and Department of Cell Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston.

[*] *Reprint requests to:* Dr. George Poste, SmithKline and French Laboratories, L328, 1500 Spring Garden St, Philadelphia, PA 19101.

tal and questions the scientific rationale of certain approaches to chemotherapy in the light of newer knowledge about the cellular composition of tumors which reveals previously unimagined degrees of complexity and diversity in the therapeutic responses of different cell populations within the same tumor.

This article provides a brief survey of current concepts of tumor cell heterogeneity and their implications for current approaches to the therapy of metastatic disease and for the development of new therapeutic agents and drug delivery systems to circumvent the problem of tumor cell heterogeneity.

## Tumor Cell Heterogeneity: A Major Obstacle to Effective Cancer Treatment

The cellular heterogeneity of neoplasms has been known since the last century, when histologic studies first identified morphologic differences among cells within the same tumor. Since then, the use of increasingly sophisticated methods, and clonal analysis in particular, has revealed extensive heterogeneity in the expression of various phenotypic properties by tumor cells in both primary and metastatic lesions in the same host. These include differences in: karyotype; antigenicity; immunogenicity; biochemical properties; growth behavior; susceptibility to destruction by chemotherapeutic drugs, radiation, hyperthermia; and the ability to evade humoral and/or cell-mediated immune reactions mounted by the host (1-6).

The exact number of phenotypically distinct clonal subpopulations present in different types of malignant tumors is not known. The number is probably very high. It is also dynamic, with the number and nature of the subpopulations changing during tumor progression in response to the varied selection pressures that act on the constituent subpopulations (3,7). Of particular importance from a clinical standpoint is that individual tumor cell subpopulations differ significantly in terms of their metastatic properties and in their susceptibilities to killing by host defense mechanisms and therapeutic modalities (1-4,8). Malignant tumors in advanced progression thus represent complex cellular mosaics containing diverse cellular subpopulations that display seemingly limitless patterns of phenotypic variation.

The issue of whether subpopulations of tumor cells with heterogeneous phenotypes coexist in the same tumor no longer merits debate. The more challenging issue is to gain an understanding of how the extensive cellular heterogeneity identified in both human and animal tumors is generated and whether it might be possible to limit the rate and the extent of phenotypic diversification to improve therapeutic efficacy.

We presently know little about when tumor cells endowed with metastatic properties first arise in malignant human tumors of different histologic origins. Are they present in certain tumors from the very outset of neoplastic conversion? What controls the fraction of metastatic cells in a tumor? Once metastatic subpopulations are present, do they have adaptive advantage over nonmetastatic subpopulations so that their contribution to the overall cell population will increase with progressive growth of the tumor? Answers to these questions are lacking.

Differences in the time at which metastatic subpopulations first emerge in primary tumors, and/or differences in the timing with which they fully express their metastatic potential, may explain the relatively consistent differences in metastatic behavior observed in human malignancies arising in different tissues (9). The factors responsible for these patterns of metastatic behavior are not known. In malignant neoplasms that carry a high risk of early metastasis, metastatic subpopulations must presumably be present very early in the life of the primary tumor. In tumors of moderate metastatic potential, the increasing risk of metastasis accompanying progressive enlargement of the primary tumor may reflect emergence of a larger number of increasingly aggressive metastatic subpopulations. In addition, the dose of tumor cells released from many tumors is known to increase with progressive enlargement (9).

Similar ambiguities apply to the emergence of tumor cells that are resistant to one or more antineoplastic agents. For example, does the evolution of such cells display a consistently different kinetic pattern in neoplasms in tumors arising in different tissues? What is the relationship between the size of the self-renewing stem cell fraction, the egress of stem cell progeny into a "differentiated" compartment with limited proliferative capacity, and the emergence of stem cells which are resistant to one, two, or multiple antineoplastic agents? Are subpopulations of resistant stem cells present prior to therapy in tumors that are classified as relatively refractory or refractory to chemotherapy? How do the duration, frequency, and sequence of exposure to antineoplastic agents affect the evolution of tumor stem cell variants that are resistant to one or more classes of antineoplastic agents? Does exposure of tumor cells to sublethal concentrations of antineoplastic agents with known genotoxic activity accelerate the emergence of drug-resistance phenotypes? What is the role of sublethal damage imposed by host response reactions in enhancing genomic instability in tumor cells? Although painstaking experimental and clinical investigations to answer these questions are in progress (4,10,11,16), it will be many years before they can be answered in suitable detail for any of the major human malignancies.

### *Genesis and Control of Tumor Cell Heterogeneity in Primary Tumors and Metastases*

Cellular diversity can arise in tumors by three nonexclusive mechanisms. First, for tumors of multicellular

origin, diversity would be present from the outset. However, even in these tumors, and certainly for tumors of unicellular origin (which is believed to be the mode of origin of most human tumors), the available evidence suggests that the principal mechanism for generating diversity is the emergence of cell variants during progressive tumor growth. The desired alternative is via fusion of tumor cells or tumor cells and host cells to create viable hybrid cells.

Nowell (12), elaborating on the concept advanced earlier by Foulds (13), proposed that generation of cell variants is an inevitable and fundamental feature of progressive tumor growth. Nowell proposed that tumor progression occurs via a series of multiple, yet independent, changes in many different cellular properties, resulting in rapid generation of clonal subpopulations with widely differing phenotypes. This diversity is amplified by the fact that tumor progression toward autonomy from host control may proceed simultaneously along many pathways and need not follow a set "program" of phenotypic change.

At any time during progression, the number of cellular subpopulations present in a tumor and their phenotypes will reflect the selection pressures encountered during the lifetime of the tumor. Selection pressures can be natural (eg, assault by host defense mechanisms; limiting nutritional conditions) or applied (eg, therapy). Even though the kinetics of the generation of cell variants may be different in tumors with different histologic origins, as well as in tumors of common histologic origin in different patients, the probability is high that by the time of initial clinical presentation most tumors will exhibit extensive cellular heterogeneity for a wide range of phenotypes (1,2,14,15,16).

Neither the nature nor the intensity of the selection pressures operating in any two hosts bearing tumors of similar histologic origin will be identical. Consequently, the panel of tumor cell subpopulations present in any two experimental animal hosts inoculated with the same tumor, or two cancer patients with ostensibly the same type of tumor, will inevitably be different.

The dynamics of tumor cell diversification during progressive tumor growth are still poorly understood. As in other examples of population dynamics, the extent of cellular heterogeneity present at any given time will reflect the balance between the formation of new cell variants with altered phenotypes and their subsequent proliferative expansion versus their elimination by host responses and/or therapy. There is evidence to suggest, however, that metastatic tumor cells may generate cell variants at higher frequencies than nonneoplastic cells (2,4,14). The formation of cell variants with altered phenotypes in mammalian somatic cell populations via classical mutations typically occurs at rates of $10^{-6}$ to $10^{-8}$ per cell per generation (17). In contrast, studies using cloned tumor cells have revealed rates of variant formation as high as $10^{-3}$ to $10^{-5}$ per cell per generation (4,14). There is a growing literature which indicates that many tumor cell clones display rapid phenotypic "drift," both in vitro and in vivo, and quickly generate progeny with altered growth rates, metastatic properties, antigenic profiles, and altered responses to therapeutic agents (2,4,14,18-20).

Analysis of the phenotypic stability of cloned tumor cells has also revealed a facet of phenotypic diversification that may be of profound significance in tumor biology and a major obstacle to the design of therapeutic strategies to limit or circumvent tumor cell heterogeneity. In 1981, experiments from the author's laboratory showed that murine B16 melanoma cell clones generated new subclones with altered phenotypes at a faster rate when grown as single clones than when cocultivated with other B16 clones (21). These observations raised the prospect that tumors might not simply be passive assemblies of cells, but interactive assemblies in which the constituent cellular subpopulations could influence each other's behavior. Similar observations describing differences in the phenotypic stability of tumor cell clones when grown singly than when cocultured with other clones have been reported by several laboratories (4,14,18-20). These observations suggest that the stability of certain phenotypic traits in tumor cells, including metastatic proficiency and responses to anticancer drugs, can vary depending on the microenvironment in which the cell resides. In heterogeneous tumor cell populations containing multiple clonal cellular subpopulations, the rate of formation of cell variants is significantly lower than when the same cells exist as single clones in isolation. Further evidence to support the role of cellular diversity in modulating the phenotypic stability of tumor cells was provided by experiments showing that events that restrict subpopulation diversity in polyclonal tumor cell populations stimulate the formation of new variants from the remaining clones (21). The generation of new variants then continues until the level of subpopulation diversity is achieved, at which the formation of cell variants again slows. Imposition of a new selection pressure to eliminate the majority of clones then initiates another cycle of rapid phenotypic diversification (21). Similar processes appear to be operating in tumors in vivo. Clonal analysis of a series of individual lung metastases produced by B16 melanoma cell clones revealed that the majority (> 80%) of metastases originated from the arrest and proliferation of a single tumor cell, but within as little as 45 days, the lesions contained a variety of phenotypically diverse clones as a result of rapid formation of variant progeny (22,23). The interpretation that rapid generation of tumor cell heterogeneity within metastases is stimulated by the limited cellular diversity present in "early" metastases is favored by data showing that similar phenotypic drift did not occur when the same tumor cell clones were cocultivated with other clones in vitro (21,22). Even more

compelling is the finding that tumor cells in metastases produced by direct inoculation of polyclonal mixtures of the same clones into the lung or by trapping polyclonal cell mixtures in the lung on the surface of microbeads failed to undergo phenotypic diversification (23), presumably because of the presence of other clones in their immediate microenvironment.

By analyzing the clonal composition of newly induced tumors after different periods of growth, we have found that the fraction of tumor clones in a tumor exhibiting "stable" or "unstable" metastatic phenotypes (when grown in isolation) changes with tumor progression (15). Murine UV-induced fibrosarcomas and methylcholanthrene-induced tumors that were excised within 3 months after their initial detection were found to contain only a very small fraction of clones with unstable metastatic properties. The fraction of unstable clones in these populations increased during further passage in culture and serial transplantation in syngeneic mice (15). Of more interest, however, was the finding that the proportion of unstable clones increased in cell populations harvested from tumors that were allowed to reach an advanced stage of progression before excision (15). These results suggest that increasingly unstable clones evolve in situ and that the proportion of such clones increases during tumor progression. As in the case of clones isolated from "early" tumors, serial passage of cells from "advanced" tumors increases the fraction of unstable clones in the population. The term "unstable clone(s)" used here refers to the expression of unstable metastatic properties in clones maintained in the absence of other clones, and does not mean that similar instability will occur when the clone exists within a heterogeneous, polyclonal tumor mass in situ or cocultivated with other clones in vitro. Examples have been identified, however, of clones isolated from "advanced" tumors that display unstable metastatic properties and which continue to generate cell variants with altered metastatic properties even when maintained in a polyclonal population (15).

The finding that interactions occurring between the clonal cellular subpopulations present in heterogeneous tumors can influence the rate at which new variant subpopulations are generated introduces additional complexity to the experimental study of tumors. The mechanism(s) underlying these fascinating events is not known. Irrespective of the mechanism involved, identification of interactions between different subpopulations from the same tumor suggests that a full understanding of the role of tumor cell heterogeneity in determining the behavior of tumors and in predicting their response to therapy cannot be achieved by simple analysis of the individual component subpopulations, but requires a more sophisticated analysis of the interactions between different tumor cell subpopulations.

### *Tumor Cell Heterogeneity and the Biological "Fitness" of Tumors*

The genesis of cellular diversity within tumors via the generation and expansion of phenotypically different tumor cell clones during tumor progression provides a powerful mechanism for increasing the ability of a tumor to survive a diverse array of destructive selection pressures. The unrelenting emergence of new clonal subpopulations of tumor cells with diverse phenotypes in response to the varied selection pressures acting on a tumor increases the probability that one or more subpopulations will possess the phenotypes needed to survive selection pressures mounted by the host and by the oncologist during cancer therapy. The genesis of cellular diversity can thus be viewed as a major factor contributing to the biological "fitness" of tumors. The term fitness is used here in the classical "Darwinian" sense to describe competence (fitness) to survive in the face of selection pressures that eliminate cells lacking the specific traits required for survival (unfit cells).

### *Autoregulation: Clonal Interactions as a Mechanism for "Protecting" Tumors Against "Domination" by Limited Number of Clonal Subpopulations*

Enhanced formation of tumor cell variants under conditions of restricted subpopulation diversity also affords tumors with a potential mechanism for regenerating cellular diversity in response to changes in cellular composition resulting from the tumor's own growth dynamics. Rapid clonal diversification under conditions of limited cellular diversity would offer a tumor protection against being "dominated" by a single clone or small number of clones. New clones might be generated in the course of tumor progression that have significant adaptive advantages over other clones and will become the dominant, or even the exclusive, subpopulation within the tumor. Clonal domination could occur via a number of nonexclusive mechanisms, eg, faster growth rate, more efficient competition for nutrients under rate-limiting conditions, or the ability to escape detection by host defense mechanisms. Irrespective of the mechanism involved, domination of a tumor by a single/few subpopulation(s) will limit cellular diversity and would thus serve as a stimulus for diversification of the dominant clone(s).

### *Clonal Interactions and Regeneration of Tumor Cell Diversity Following Therapy*

The ability to rapidly generate new subpopulations of tumor cells with novel phenotypes under conditions in which the overall level of subpopulation diversity is restricted would ensure that a tumor would reduce the

risk of becoming dominated by a small number of subpopulations or even a single subpopulation. Tumor cell heterogeneity reduces the likelihood that a destructive assault mounted against the tumor by host defense mechanisms or by the oncologist will be successful in eliminating all of the subpopulations present. On the other hand, the stimulation of clonal diversification caused by events which reduce subpopulation diversity would provide a potent mechanism for the relentless generation of new variants each time a tumor is exposed to an assault that destroys a significant fraction of the cellular subpopulations present.

The most challenging, and certainly the most disturbing, question arising from this proposal concerns the potential effect of therapy (single/multiagent; single/multimodality) on the dynamics of tumor cell subpopulations. If a particular therapy[2] were to kill the majority, but not all, of cellular subpopulations in a heterogeneous, polyclonal tumor (as is typically the case), the surviving subpopulations might be rendered phenotypically unstable because of loss of the regulatory interaction between constituent subpopulations (fig 1). Therapeutic restriction of tumor cell subpopulation diversity in a polyclonal cell population in which the level of clonal diversity is sufficient that formation of new clonal variants is at a low level would stimulate rapid formation of new tumor cell variants from the surviving subpopulations. Generation of variants would then continue until a sufficient level of subpopulation diversity imposed a new plateau as a result of "interactions" occurring between the cell subpopulations and the rate at which new variants emerge would fall (fig 1). Exposure of the tumor to further treatment regimens would then repeat the cycle unless a therapeutic regimen could be identified that was able to eliminate the surviving subpopulations before they had time to generate new variants.

If this situation is occurring in human neoplasms, new therapeutic strategies will need to be explored with the objective of frustrating the phenotypic instability in tumor cell subpopulations that survive a previous treatment protocol. One approach would be to reduce the time interval between successive treatments with different agents. We have coined the acronym FAST (Fast Accelerated Staging of Therapy) to describe this strategy (14,15,24), in which a series of different treatment protocols would be used in rapid succession to attempt to kill the subpopulations that survive the previous treatment before they can generate large numbers of new variants and to kill any new variant subpopulations as soon as they emerge (fig 2). This contrasts with the practice used in many clinical situations in which subsequent treatment regimens are often not employed if the first treatment protocol appears successful in eliminating clinically detectable tumor and additional treatment is implemented only when evidence of a recurrent tumor is detected. By the time such recurrent lesions become detectable clinically, the probability that the surviving subpopulations have diversified is high, therefore creating a recurrent lesion containing a new set of tumor cell subpopulations which may have widely differing therapeutic sensitivities (fig 3).

The heterogeneous nature of the responses of malignant tumor cell subpopulations to cytotoxic drugs and other therapeutic modalities makes it unlikely that a single treatment regimen will be able to kill all the cells in a tumor, even if several agents are used simultaneously. This problem has been all too familiar to clinicians for a long time. Treatments are available

[2]The terms therapy and treatment used here do not refer to any specific anticancer agent or modality. The terms apply to multiagent/multimodalities as well as single-agent/modality treatment protocols.

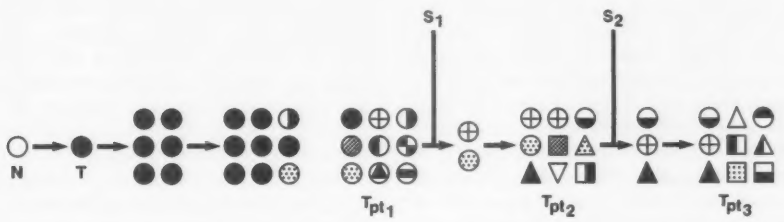


FIGURE 1.—Possible effect of potent selection pressures in causing cycles of clonal restriction and diversification in tumors. Following conversion of a normal cell (N) to a tumor cell (T), additional tumor cell variants arise (depicted by the different symbols) to create a heterogeneous, polyclonal lesion, which eventually reaches a plateau phase ($T_{pt_1}$), at which time the rate of formation of new variants is reduced (*see* text). Exposure of a heterogeneous, polyclonal tumor cell population of this kind to a selection pressure ($S_1$) that eliminates the majority of clones causes rapid formation of a new panel of clonal variants from the surviving clones. With time, sufficient subpopulation diversity is again achieved to establish a new plateau phase population ($T_{pt_2}$). Exposure of this population to a new (different) selection pressure ($S_2$) then elicits another cycle of clonal loss and phenotypic diversification of the surviving clones. Fig reproduced from ref 15.

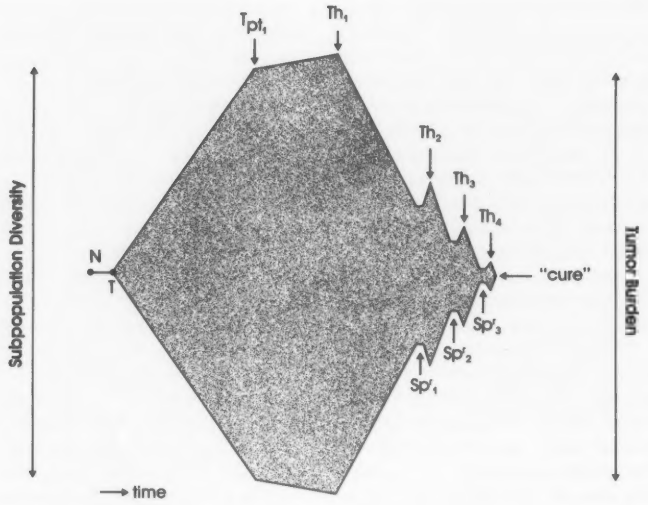


FIGURE 2.—Schematic illustration of proposed FAST cancer treatment protocol. Following initial transformation of a normal cell (N) to establish an initial focus of tumor cells (T), new variants are generated, creating a heterogeneous lesion of diverse subpopulations that eventually establish a "plateau" state ($T_{pt}$). Therapy ($Th_1$) reduces the subpopulation diversity and cell mass. The restriction of diversity may stimulate surviving resistant subpopulations ($Sp^r_1$) to rapidly generate new metastatis variants. However, the second treatment regimen ($Th_2$) is given very shortly after the first treatment protocol, even though a residual tumor burden may not be evident clinically. By repeating the process and reducing the interval between successive treatment regimens ($Th_1$-$Th_3$), it is intended that each treatment would kill susceptible subpopulations that survive the preceding treatments ($Sp^r_{1-3}$) before they generate a large number of new variants, some of which might prove resistant to all available therapies. It may thus be possible to either eradicate tumor cells entirely or reduce the residual tumor burden sufficiently to allow host defense mechanisms, perhaps augmented by biological response modifier therapy, to kill any remaining tumor cells. Fig reproduced from ref 15.

which are effective in killing the majority of tumor cells within a tumor, and the remaining tumor burden is often sufficiently small that it cannot be detected clinically. In many situations, further treatment regimens may not be implemented routinely following completion of treatment that eliminates clinically detectable tumor. A new regimen is implemented only when the patient presents some time later with clinical evidence of recurrent disease. However, the data discussed in this article suggest that by the time recurrent disease is diagnosed and a new cycle of therapy initiated, the tumor cells present in the recurrent lesion would be phenotypically very different from those in the original tumor due to rapid formation of new variant subpopulations induced by the restriction in subpopulation diversity caused by the first treatment regimen. The subpopulations of tumor cells present in the recurrent lesions will not only be resistant to the agent(s) used in the first treatment protocol, but will also have a high probability of being resistant to other agents.

Research on these questions is only just beginning, and considerably more knowledge is needed before the question of accelerated staging of successive treatment cycles could be considered in the clinic. Similarly, in raising the possibility that therapy may stimulate phenotypic diversification and facilitate emergence of increasingly aggressive tumor cell variants, it is not proposed that current therapies be withheld or abandoned. Further experiments are needed to determine the effect of different therapeutic modalities, and their staging, on the phenotypic stability and metastatic behavior of tumor cell subpopulations in neoplasms containing different numbers of identifiable subpopulations. The technical difficulties of such experiments are not minimized. Most rodent tumors are unsuitable for evaluating the FAST protocol, and entirely new test systems will need to be devised.

Even if the FAST protocol or analogous strategies to limit rapid phenotypic diversification of tumor cells following subcurative therapy have logical origins in current concepts of the cell biology of metastatic disease, their practical implementation is fraught with great difficulty. The substantial toxicity associated with many current cancer treatment protocols dictates that clinical management of patients subjected to ever more aggressive and prolonged treatments would be taxing, both in terms of risk to the patient and the need for intensive care and other support facilities. Patient com-

pliance would also be a major problem. In addition, in the absence of diagnostic and prognostic criteria to accurately identify those patients who will present with recurrent disease after completing the initial treatment regimen, such patients would be exposed to an unnecessary therapeutic assault. Although the same dilemma applies to current adjuvant therapy, the greater intensity of the therapeutic intervention proposed in the FAST protocol could be viewed by many as an unacceptable risk.

### Need for New Strategies to Circumvent or Limit Tumor Cell Heterogeneity

Recognition of the extensive, and perhaps even radical, phenotypic heterogeneity exhibited by malignant tumor cells creates an urgent need for new strategies to identify traits shared by all tumor cells which could be used therapeutically to circumvent the heterogeneous responses encountered with current antineoplastic agents. The prospects of identifying such traits, which should also be lacking in normal cells, are considered remote. The only tumor cell property described to date that even approximates the definition of a "universal" trait that could be exploited therapeutically is the highly efficient recognition and destruction of tumor cells by activated macrophages. Macrophage-mediated cytotoxicity is highly selective for tumor cells and is not compromised by heterogeneity in tumor cell growth rates, proliferative status, antigenicity, metastatic abilities, or cellular resistance to drugs, antibodies, lymphocytes, or natural killer cells (25-28). Unlike acquisition of resistance to chemotherapeutic agents which occurs in tumor cells at a relatively rapid rate (10), the relative infrequency of tumor cell variants resistant to activated macrophages is striking (27). These observations have prompted a number of investigators to explore new therapeutic approaches for augmenting macrophage-mediated destruction of metastases. A comprehensive analysis of this strategy has been made by Fidler et al (27) and Xu et al (28), using liposomes as a carrier system for the selective delivery of diverse activation agents to macrophages in vivo. Considerable success has been achieved using this approach to treat established lung and liver metastases in rodent tumor models. Although these studies demonstrate the effectiveness of activated macrophages in circumventing the problem of tumor cell heterogeneity, the efficacy of this modality in treating substantial metastatic burdens has still to be established. If the ratio of macrophages to tumor cells required for optimal macrophage-mediated tumoricidal activity in vivo is similar to that operating in vitro, then, even allowing for maximum recruitment of activated monocytes from the bloodstream into extravascular metastases, there are probably insufficient macrophages to destroy metastatic

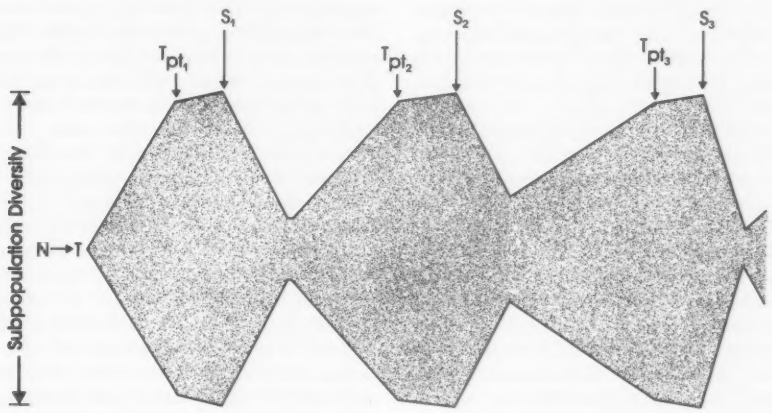


FIGURE 3.—Schematic illustration of the possible effects of extended staging of successive treatment regimens on subpopulation diversity in neoplastic lesions. Following initial transformation of a normal cell (N) to establish an initial focus of tumor cells (T), extensive clonal diversity is generated, creating a heterogeneous lesion of diverse subpopulations that interact and reach a "plateau" state ($Tpt_1$). Treatment ($S_1$) destroys some subpopulations. This restriction in subpopulation diversity will stimulate the surviving subpopulation to generate new variants, culminating in a new "plateau" state ($T_{pt_2}$). By this time, recurrent tumor growth is detectable clinically and a second, different treatment regimen ($S_2$) is initiated, and another cycle ensues. This process will continue until either the patient dies of a tumor refractory to multiple successive treatments or a therapy eradicates the entire remaining tumor burden. The term therapy does not refer to any specific anticancer agent or modality. The illustrated sequence of events is applicable to treatment regimens using any agent or modality either alone or in combination which fail to kill all of the tumor subpopulations present in the lesion. Fig reproduced from ref 15.

burdens in man that exceed $10^{10}$ tumor cells (29). Also, heterogeneity in the recruitment of macrophages into individual metastases in the same target organ (30) may also compromise the efficacy of nonspecific immunotherapy protocols of this kind.

Therapeutic regimens designed to stimulate the antitumor properties of macrophages will therefore have to be used in combination with other treatments such as chemotherapy, which would be used to reduce the "bulk" tumor burden to a level sufficient to allow activated macrophages to kill the surviving tumor cells. Nonetheless, initial results with liposome-mediated delivery of activation agents to macrophages have been more successful than any other approach in recent years in treating established metastases, and further studies are undoubtedly warranted. Studies to evaluate the efficacy of liposome-encapsulated immunomodulators in combination with chemotherapeutic regimens in treating graded metastatic burdens in experimental animals are currently in progress in several laboratories.

The scale of the phenotypic heterogeneity exhibited by tumor cells may be sufficiently large, even in very early lesions, that the search for therapeutic agents to circumvent the repertoire of cellular diversity may be unrealistic. An alternative approach would be to develop methods to limit tumor cell heterogeneity, either by reducing the rate of formation of cell variants or by favoring the emergence of new cell variants with defined phenotypes (controlled or directed heterogeneity). By constraining cellular diversity, and/or by directing phenotypic evolution along more predictable pathways, it might be possible to use existing agents in new combinations for therapeutic gain.

The ability to direct the phenotypic diversification of tumor cells in a more controlled and predictable fashion is an appealing concept and also introduces the possibility that the changes induced in the tumor cells could serve as new therapeutic targets. However, practical translation of this concept to even experimental settings must await a more detailed understanding of the mechanisms responsible for the phenotypic plasticity exhibited by tumor cells. As mentioned, the rate of formation of cell variants in many tumor cells appears to be higher than that expected on the basis of genetic mutations, suggesting that epigenetic mechanisms are also operating. Several nonexclusive mechanisms for epigenetic variation could be responsible, but experimental evidence is lacking. The only pertinent information to date concerns observations showing that treatment of tumor cells with agents such as 5-azacitidine that induce DNA hypomethylation can produce marked alterations in tumor cell properties, including loss of metastatic properties (31-34). However, the question of whether alterations in DNA methylation per se are responsible for these changes has yet to be established. Even if this were so, the exercise of defining the genomic locus, or loci, involved in the modulation of metastatic properties is formidable. However, such experiments at least illustrate that an understanding of the mechanisms responsible for the phenotypic lability of tumor cells could create new opportunities for therapeutic manipulation of phenotypic expression in these cells, including opportunities for driving tumor cells into phenotypic pathways that result in loss of "dangerous" phenotypes such as proliferative ability, metastatic competence, and drug resistance.

## Site-Specific Drug Delivery (Targeting) and the Therapy of Metastatic Disease

Considerable ingenuity has been devoted over the past decade to the development of systems for improved drug delivery in cancer treatment. These range from efforts to reduce toxicity using rate-controlled release preparations and implantable devices, to improving the effectiveness of antitumor drugs by using new formulations with improved pharmacokinetic and/or pharmacodynamic profiles, to more ambitious efforts to "target" drugs to tumor cells while leaving normal cells unharmed.

The clinical appeal and the therapeutic advantage of targeted drug delivery to selected cell types in the body do not need to be stated. Cancer probably represents the most obvious, and is certainly the most quoted, example of a disease in which targeted drug delivery and selective destruction of tumor cells would produce truly dramatic therapeutic gains. It is thus no surprise that this therapeutic goal has been the common focal point for myriad experimental approaches to targeted drug delivery in vivo, ranging from particulate carriers (liposomes, nanoparticles, microspheres) to macromolecular carriers such as antitumor antibodies linked to drugs, radioisotopes, or bacterial and plant toxins. Research on this topic has increased significantly in recent years, stimulated by advances in our understanding of the surface properties of different cell types and the possibility of using monoclonal antibodies and other ligands to target drugs to tumor cells bearing unique surface determinants.

Despite its obvious theoretical attractions, selective drug delivery to tumor cells, and to metastatic lesions in particular, poses substantial technical difficulties (35-38). Although the media hyperbole generated in recent years by numerous premature, and unwarranted, claims regarding the imminent success of targeting drugs and toxins to tumors using monoclonal antibodies, liposomes, or the latest candidate for the "magic bullet" award may have resulted in the death of many trees, they have yet to make a significant contribution to improving the life of cancer patients with metastatic disease. Indeed, preclinical information on their effectiveness in treating metastatic disease in animal models is scant. Worse still, in too many instances, this crucial requirement has never been tested with therapeutic efficacy being tested only against ascites tumors growing ip or well-vascularized tumors implanted sc. Ip therapy of a minimal burden of tumor cells growing in an ascites form

in the peritoneal cavity is of little relevance, since the drug or the drug carrier gains immediate access to tumor cells without interference from anatomic barriers. Similarly, well-vascularized, transplanted tumors growing sc or im, although more difficult to eradicate than ip tumors, fail to provide a sufficiently demanding model for evaluating the effectiveness of agents in treating metastases. All animal tumors are flawed in some respect as models of human neoplasms. This does not mean that efforts should not be made to use models that share as many features as possible with human malignancies. We consider that this requires that drug delivery systems be evaluated for their activity against established metastases in an adjuvant therapy setting comparable to that undertaken in the clinic following removal/reduction of the primary tumor. The rationale for proposing that experimental therapies be tested for activity against *established* metastases is not based on a belief that the therapeutic responses of cells in metastases may differ from tumor cells in the primary lesion. There is no a priori reason to assume that the chemosensitivities of metastatic tumor cell subpopulations cannot be determined by assaying the response of a heterogeneous tumor cell population containing both metastatic and nonmetastatic cell subpopulations implanted sc or im. The rationale for using metastatic tumor models is to confirm that the disposition and pharmacokinetics of a drug and its delivery system are consistent with achieving therapeutic drug concentrations in organs typically affected by metastases in the neoplasm of interest. Since metastases in the same host vary significantly in their size, growth rates, cell growth fraction, and vascular supply, testing in metastatic models also yields useful information about the efficacy of a drug delivery system in circumventing these factors (35).

Successful development of a site-specific drug delivery system for the therapy of any disease demands a detailed appreciation of the pathogenesis of the disease and the factors that limit the efficacy of current treatment. It is therefore unfortunate, even tragic, that so much of the extensive literature on drug targeting in which improved cancer therapy is cited as the principal objective should fail to consider such fundamental issues as tumor cell heterogeneity, the disseminated nature of metastatic disease, the permeability characteristics of the microcirculation in different body organs, and the risk of novel iatrogenic toxic effects arising from the altered disposition and retention of carrier-associated drugs.

Effective drug targeting in cancer therapy requires that, at a minimum, the approach is capable of achieving selective drug localization at body sites where tumor cells reside and releasing drugs in an active form at a suitable rate to kill tumor cells while producing minimal effects on normal host cells. Ideally, normal host cells should be spared completely if the delivery system is designed to target drug selectively to tumor cells. In the ultimate form of drug targeting, not only would the drug be delivered selectively to tumor cells, it would also be directed to specific intracellular sites to maximize its effectiveness.

Targeting of therapeutic agents to specific cell types in vivo requires successful completion of several independent steps: access to the appropriate target cell; recognition and selective interaction with the target cell; and selective uptake by the target cell with little or no uptake by nontarget cells. In addition, the drug and its carrier must not produce unacceptable levels of toxicity, and the drug must remain associated with the carrier for sufficient time to enable therapeutically effective drug concentrations to be delivered to the target cells. Finally, if widespread clinical use and commercial development are to occur, the carrier system must be pharmaceutically and clinically acceptable with respect to formulation homogeneity, stability on storage, sterilization, cost of manufacture, and ease of administration.

Iv drug administration presently offers the only practical route of administration for most agents used in treating metastatic disease. The localization and retention of iv administered drug carriers in tumors administered in vivo are determined by the anatomic location(s) of the tumor(s); tumor blood supply; the behavior of the carrier in the systemic circulation; and the structure and permeability of the tumor microvasculature (19,30). For solid tumors and their metastases located in extravascular tissues, targeted drug delivery to tumor cells requires that the drug-carrier complex must leave the circulation. For carriers equipped with cell-recognition ligands such as antitumor antibodies, retention will be influenced by the distribution of antigen-positive tumor cells and the properties of the target antigen (35,42-45).

Whole-body scintigraphy reveals marked variation in the efficiency with which antitumor antibodies localize in animal and human tumors (39,40). Impressive reports of antitumor monoclonal antibodies that localize in tumors in vivo at concentrations of fivefold or greater than in normal tissues are now commonplace (39,40). However, reports are equally common in which monoclonal antibodies with similar high affinities for tumor cells in vitro fail to label in tumors in vivo (39,40). Research using monoclonal antibodies is still in its infancy, and further analysis of the effect of antibody class, the properties of the target antigen, and the cytotoxic agent coupled to the antibody, and the dose, route, and schedule of antibody administration may yield important therapeutic gains. It is important, however, that such studies also evaluate the efficiency of antibody localization and retention within metastases rather than in large tumor masses implanted sc or im.

Effective drug delivery by antitumor antibodies or other targeting ligands requires that they penetrate uniformly throughout the tumor mass. Autoradiography of histologic sections of tumors excised after iv administration of radiolabeled antibodies or antibody-conjugates reveals considerable variation in labeling patterns in different tumors (35,39,40).

Vascular hemodynamics and microvessel permeability are of obvious importance in determining the uptake of circulating drug-carrier complexes by tumors. Total blood flow, rates of perfusion, and vessel permeability may vary significantly in different regions of the same tumor (35). The vascular supply in tumors transplanted sc or im may differ substantially from metastases that arise from them. For example, implantation of a large bolus of tumor cells into host tissue disrupts the local microvasculature and enhances vascular permeability at the injection site. The latter persists until vascular repair is achieved within 2–3 days, and allows ready penetration of iv injected materials at these sites during this hyperpermeable phase. Also, the blood supply of transplanted tumors is established by emigration of new capillary sprouts from surrounding host vessels. These sprouts are highly permeable and allow virtually unlimited passage of materials, including erythrocytes, into the extravascular tissue (29,35). Penetration of liposomes, antibodies, or other circulating materials into transplanted tumors may thus be artificially high and provide little or no insight into the behavior of these materials in the vascular bed of human tumors or spontaneous animal tumors in which the vascular supply will evolve in an entirely different fashion in concert with progressive enlargement of the tumor cell population (29,35).

In contrast to antibodies and other macromolecular carriers whose initial distribution is uniform throughout the capillary circulation, the disposition of particulate carriers injected iv is nonuniform and is determined by particle size, vascular anatomy, and interaction with the reticuloendothelial system (RES) (35,38). Particles of 0.1–5.0 μM diameter injected iv or intra-arterially are cleared rapidly by macrophages of the RES in the liver, spleen, and bone marrow and circulating blood monocytes (35). Clearance kinetics are influenced by particle size, aggregation, surface charge, and composition. These parameters also affect the fraction of total particles cleared by RES components in the liver versus the spleen and the bone marrow. Irrespective of which site dominates, between 80% and 95% of the total particles injected are typically taken up by the RES (35).

Rapid clearance of small particles (0.1–5 μM diameter) by the RES is a major obstacle to targeting these structures to other sites such as metastatic foci. Efforts to alter this disposition pattern by constructing particles with prolonged circulating half-lives have been unsuccessful (35). Such particles still localize in the RES, albeit more slowly (37). The slower clearance of such particles may enable them to be used as rate-controlled drug-release devices in cancer therapy, particularly for labile drugs that are rapidly inactivated or degraded by blood components. This requires that particles can be constructed with reproducible permeability characteristics to ensure that the rate of drug release is consistent with sustaining therapeutic levels of drug in the blood. Ironically, the search for highly stable liposomes and other particles with very low permeability characteristics and long circulating half-lives may be of limited value if the rate of drug release is so slow that therapeutic drug levels are never achieved (35).

Blockade of the ability of the RES to clear particles by predosing with empty particles without drug before injecting of particles containing drug has been advocated as a way of diverting particulate drug carriers away from the RES. Although this strategy enhances liposome localization of small (< 800A), but not large, particles in some, but not all, tumors (35), this technique has no effect on liposome localization in metastases (29). Even under conditions of RES blockade, a higher concentration of liposome-associated drug may still accumulate in macrophages of the RES than in unblockaded animals treated with free drug. This may be of little consequence for many classes of drugs, but for highly cytotoxic agents of the kind used in cancer therapy, this can cause destruction of the RES (*see* below).

The key assumption in proposals to use liposomes to target drugs to tumors is that these structures can extravasate to reach tumor cells in the extravascular compartment. There is no evidence to support this assumption. Extravasation of liposomes occurs only in sinusoidal capillaries of the liver, spleen, and bone marrow that lack a continuous endothelium and an underlying basement membrane, and liposome extravasation has not been detected in tissues lined by continuous and fenestrated capillaries which possess an uninterrupted basement membrane (38). Extravasation of circulating liposomes does not occur, even at sites of inflammation in vessels that are highly permeable to macromolecules (38).

The failure of liposomes and similar particulate carriers to extravasate on a scale consistent with therapeutic benefit was probably predictable on mechanical grounds and from known data on the restricted extravasation of materials from the microcirculation. Proponents of the use of particulate drug delivery systems argue that the concept may still be feasible because structural defects in tumor microvessels will permit their escape into extravascular tissues. That the absence of a continuous basement membrane in certain tumor capillaries and the presence of blood channels lined by tumor cells rather than endothelial cells will increase the extravasation of materials from the circulation is not disputed (41). What is not known, and cannot be predicted with any accuracy in any particular tumor, is what fraction of the tumor vasculature is composed of such vessels. Even within the same tumor, structurally normal capillaries exist in close proximity to abnormal vessels (41). Changes in vascular permeability within tumors should not be overstated; they are still relative, and unlimited permeability is found only in microvessels displaying marked structural changes (35,41). Free access of circulating materials to the extravascular interstitium in tumors is not found, even for serum proteins (41).

Advocates of the value of particulate carriers for drug targeting to tumors speculate that ways may still be found to render vessels permeable to such carriers. Such methods could probably be devised, but for what purpose and at what risk? It is difficult to envisage how selectivity could be imposed on the process and how it could be confined to the microcirculation of interest. Vessels "permeabilized" in this way may also be highly leaky for a wide array of macromolecules that do not ordinarily penetrate into the extravascular space (and should not). In my opinion, such proposals are naive and pose the risk of significant toxicity.

The majority of the proposed systems for targeting drugs to cancer cells also fail to address the problem of tumor cell heterogeneity. Although selective targeting of drugs to tumor cells would produce major therapeutic gains by reducing toxicity to normal tissues, limitations in current antitumor drugs dictate that survival of drug-resistant tumor cells will continue to be a major cause of treatment failure.

Tumor cell heterogeneity also has profound implication for the use of antibodies as targeting ligands, either as direct conjugates to drugs and toxins or as cell recognition ligands coupled to particulate carriers. Qualitative and quantitative differences in antigen expression have been identified between tumor cells in the same primary tumor, between cells in the primary tumor and metastases, and between cells in individual metastases arising from the same primary tumor (35,42-45). Effective targeting will thus likely require a "cocktail" of different antibodies, and different "cocktails" may be needed to treat tumors in different patients. If the latter applies, prospects for commercial development and the routine clinical use of this approach are bleak, since the antigenic profile of every patient's metastases cannot be defined. In addition, the target antigen(s) selected must be expressed by the clonogenic stem cell fraction in a form accessible to antibodies, and binding to the target antigen must allow subsequent drug delivery into the cell (35).

The availability of a large panel of antitumor antibodies is no guarantee of success. Antigenic modulation and/or immunoselection of antigenic-negative tumor cells remain real risks. The factors regulating antigen modulation after exposure to antibody are poorly understood. Some antigens modulate slowly or not at all, while others modulate rapidly (35). Also, surface antigens that are unaffected by exposure to a single antibody may modulate when exposed to multiple antibodies directed against unrelated antigens (35).

The signal transduction mechanisms involved in the internalization of antigen-antibody complexes are poorly understood. The complexity of this event is illustrated by studies on the internalization of drug-containing liposomes bearing monoclonal antibodies directed against different surface antigens on mouse B and T lymphocytes, in which liposome binding and internalization by the same cell type differed significantly depending on the target antigen used (46). Also, monoclonal antibodies directed against different epitopes on the same target antigen induced different internalization responses (46). It may thus be necessary to establish that targeting ligands not only display suitable specificities, but can also evoke internalization of the cell receptor-ligand-drug (carrier) complex. Once internalized, effective therapy requires that the drug/toxin can dissociate from the complex and exit from the endosome.

In targeting to surface determinants that are modulated by shedding of ligand-receptor complexes from the cell surface, effective therapy will require that the drug be released from the complex, preferably before shedding occurs, and that the concentration of drug in the biophase, and its residence time, are sufficient for killing. Selectivity is compromised, however, since drugs released extracellularly will also affect normal cells in the vicinity.

Free antigen blockade poses another potential drawback to antibody-mediated drug targeting in vivo (35). Even if blocking antigens are not present at the onset of therapy, tumor cell destruction induced by initial treatment may quickly elevate the level of free antigen in the circulation and reduce the effectiveness of subsequent treatment cycles.

The disposition, pharmacokinetics, and metabolic fate of drugs administered in association with a carrier may differ substantially from conventional formulations of the same agents, and the risk of novel toxic effects must therefore be considered (35). Perhaps the most obvious example concerns the use of liposomes and other particulate carriers to deliver anticancer drugs. By delivering high concentrations of cytotoxic drugs to mononuclear phagocytes in the blood and the RES, this approach may induce toxic ablation of a vital element of host defense. Inhibitors of DNA synthesis might be expected to have little toxic effect on nondividing macrophage populations, but drugs that impair RNA and protein synthesis may be toxic to these cells. Recent studies have shown that this fear is justified. Systemic administration of several antitumor drugs encapsulated within liposomes was found to enhance the metastatic spread of mouse tumors (47). This effect was not induced by liposomes injected sc or im and it was reversed by injection of syngeneic macrophages 12 hours after each treatment cycle, indicating that the iatrogenic enhancement of metastases was probably caused by toxic destruction of mononuclear phagocytes. The case for using liposomes or other particulate carriers for drug delivery in cancer treatment may thus be seriously flawed if the drug in question can destroy host macrophages.

Drug-antibody conjugates and immunotoxins are not exempt from this complication. Scintigraphic analyses of the disposition of radiolabeled antitumor antibodies in both animals and man indicate that even when significant labeling of the tumor is achieved, high levels of non-

specific antibody uptake by the liver and spleen are also occurring (39,40). Chemical modification and/or denaturation of antibodies during coupling to drugs or toxins, and physical changes such as antibody aggregation, can also predispose to clearance by the RES (35).

In our opinion, the potential risk posed by drug carriers that show appreciable localization in the RES is of sufficient magnitude to preclude their clinical use as carriers for cytotoxic antineoplastic drugs until extensive toxicology studies are completed to show that ablation of the RES is not induced by the specific drug(s) to be used in clinical studies. Multiple dosing with particulate carriers for long periods also presents the risk of additional RES toxicity induced by the carrier itself (29).

## Search for Improved Antineoplastic Agents: The Need for New Screening Systems and a Greater Emphasis on Histiotype-Specific Agents

For almost 30 years, the search for new antineoplastic agents has been pursued primarily under the aegis of the National Cancer Institute (NCI) and the European Organization for Research on Treatment of Cancer (47–52). The strategies adopted by these organizations have relied extensively on large-scale, random screening of materials with diverse chemical structures and biological origins to detect agents able to kill a series of murine tumor cell lines in vitro and/or in vivo. Since 1975, the NCI has also screened materials for cytotoxic activity against a panel of human tumor xenografts grown in nude mice. This modification was introduced in an effort to identify agents that might be of clinical value, yet lacked activity against murine tumors. Similarly, the decision to undertake the ongoing prospective evaluations of the predictive value of human clonogenic tumor stem cell assay in detecting novel antineoplastic agents is based on the same logic. Each of these approaches is designed, however, to identify agents with activity against histologically diverse neoplasms. Unfortunately, the experience of the last three decades suggests that this type of "panaceamycin" will not be found easily.

By using screening assays that detect antineoplastic agents with a "broad spectrum" of cytotoxic activity against tumor cell lines of diverse histologic origins, is it possible that we are inadvertently selecting for agents that lack the ability to act against biochemical targets that may be more prominent in tumor cell populations in situ and which would also be expected to vary significantly in tumor cells arising in different tissues? The same risk may also apply in overlooking agents that might be effective in circumventing the cellular mechanisms that account for the intrinsic refractoriness to and/or acquired resistance exhibited by certain tumor cells to various antitumor agents. For example, the biochemical pathways responsible for the refractoriness of different tumors are presumably linked closely to phenotypic traits that are unique to specific cell lineages and/or particular states of cell differentiation. Such properties might be lost relatively rapidly when the cells are removed from the tumor and grown as serially propagated cell lines in vitro or in vivo. For example, human colorectal adenocarcinomas are refractory to the cytotoxic action of a large number of the current chemotherapeutic agents, yet most human colon cell lines resemble cell lines from other tumors in being susceptible to these agents both in vitro and when implanted into nude mice. Could this discrepancy reflect that the properties of neoplastic colon epithelial cells that render them refractory to drug action in situ are readily lost in culture?

These considerations have stimulated a growing debate as to whether the search for improved anticancer agents might be more productive if the emphasis were shifted to seek agents for the therapy of specific neoplasms using new screening systems that employ tumor cells of the same histologic origin as the target neoplasm (histiotype-specific screening).

The choice of target tumor cells for drug screening systems is not simple. The scale and cost of large-scale screening and the 10–15-year period required to allow sufficient clinical studies to assess the value of a screen in detecting clinically active agents (48,50) mean that a decision to add or discard a particular cell system requires extremely careful review. Not surprisingly, opinion differs as to the value of the current NCI screening strategy. Supporters indicate that most of the currently available antineoplastic agents were detected using the current screens. Critics point to the lack of effective agents for treatment of the major human malignancies, despite screening several thousands of new materials each year for > 20 years, as evidence that new screens are needed. There are no simple answers in this debate. It is insufficient, however, for supporters of the current system to argue in favor of maintaining the status quo merely because current screens have demonstrated some worth, no matter how small, and the new systems are unproven. This fails to address the issue of the overall efficiency of the current screening strategy as measured by the yield of clinically effective agents; for the most important human tumors, the yield has been extremely low. The question of whether this is because such agents are extremely rare or whether current screens are inadequate can be resolved only by adopting new screening procedures to replace or supplement current approaches.

The NCI is considering a major change in its screening strategy for antineoplastic agents. It has been proposed recently that the screening undertaken by the Developmental Therapeutics Program of the Division of Cancer Treatment be switched from a "compound-oriented" effort using implanted murine tumors and human xenografts to a "disease-oriented" strategy in which human tumor cell lines of differing histologic origin would be used to identify agents with "histiotype-specific" activities (53). In this strategy, agents showing selective activity against specific human tumor cell lineages would

be accorded high priority for accelerated preclinical pharmacology and toxicology testing and would be transferred to clinical trials without additional testing against in vivo human or rodent tumor models. Other agents showing less selective activity against a broad panel of human tumor cell types in vitro would undergo additional in vivo testing against human tumor xenografts before commitment to preclinical development.

The logic for the adoption of histiotype-specific screening systems in the search for antineoplastic agents is not disputed. However, the success of this strategy will depend crucially on the properties of the human tumor cell populations that are selected as the screens. Analysis of the performance of the three human tumor cell lines used as xenografts in the current NCI drug screening program indicates that their value in detecting drugs with clinical activity was no greater than the P388 mouse leukemia cell prescreen and the standard panel of five murine tumors in vivo (48). Similarly, examples abound in the literature of cytotoxic agents that are effective against a broad range of established human tumor cell lines, but have little or no clinical activity against tumors of similar histologic origins. These problems indicate the need to consider how far cell lines may undergo significant phenotypic change during prolonged cultivation in vitro and have minimal relatedness to tumor cells in situ. In contrast, freshly isolated and/or low-passage tumor cells might retain the "biochemical profile" of tumor cells growing in a patient and thus offer a more relevant system for detecting new histiotype-specific antitumor agents. Resolution of this issue is fundamental to the use of human tumor cells in drug screening programs. Unless the phenotypic similarity of human tumor cell lines used for drug screening to tumor cells in specific human malignancies can be confirmed, we are at risk of continuing to evaluate the therapeutic responses of established cell lines that may have little or no relationship to the properties of tumor cells in situ or low-passage tumor cells isolated from freshly excised neoplasms. Definition of the phenotypic changes occurring in tumor cells from human tumors of defined histologic origin when cultured in vitro will require careful examination of a broad panel of phenotypic markers that are known to be expressed by the cells in the stem cell compartment of tumors in situ. The acquisition of such information is only in its infancy, and the technical sophistication and relevance of the exercise will, of course, change with the validation and introduction of new phenotypic markers. As in so many aspects of modern research on metastasis, the task is tedious and will require considerable resources. Until this information is obtained, however, we will not be able to answer the question of whether the established tumor cell lines that presently dominate many aspects of cancer research have any meaningful relationships to tumor cells present in tumors of comparable cellular origin in situ.

Two aspects of phenotypic regulation in cultured human tumor cells merit detailed investigation in the design of improved assay systems for drug screening. The first concerns the question of how far the properties of self-renewing stem cells in specific tumors are influenced by tumor cells in the non-stem compartment and whether the absence of the latter in established cell lines (54) results in a proliferating tumor cell fraction that is a poor indicator of stem cell behavior in situ. The second, and related issue, concerns the effects of growth in vitro in causing phenotypic drift in tumor cells, over and above any phenotypic change(s) imposed by the absence of the non-stem cell component. Without such analyses, there is no guarantee that the cells used as histiotype-specific screens in vitro will exhibit phenotypic traits relevant to the therapeutic responses of tumor cells in specific neoplasms in situ.

Immortalized cell lines offer the apparent advantages of rapid growth in vitro and ease of cultivation. Most, if not all, of the cells in established lines have self-renewing proliferative capacities, and thus represent a population of close to 100% stem cells (54). Since stem cells are the primary target for therapy, the use of cell lines in drug screening would seem logical and also offer the advantage of eliminating any spurious effects produced by drug action on non-stem cell populations. However, this interpretation assumes that the properties of self-renewing tumor cell subpopulations in cell lines mimic stem cells in tumors. This is by no means certain and ignores several potentially important questions. For example, have the self-renewing tumor cells in any given cell line completely lost the capacity to generate "differentiated" progeny with limited proliferative capacities? How far does the lack of a non-stem cell compartment in a cell line deprive the proliferating "stem cell" fraction of a source of paracrine and autocrine signals that might be important in regulating the size of the stem cell fraction, and its proliferative status? These events, in turn, would be expected to influence the opportunities for mutational events to occur to generate cells with drug-resistance phenotypes (10,55). Similarly, how far does the complete or virtual absence of egress of stem cell progeny into a non-stem cell differentiation pathway alter their responses to various drug classes?

In addition to the lack of information on how far imbalance in the stem cell:non-stem cell ratio in established cell lines and the resulting distortion of any "interactions" which might be occurring between these fractions in tumors in situ may render cell lines unsuitable to serve as in vitro "caricatures" of tumor cells in situ, we are equally ignorant about the nature and the extent of phenotype modulation which accompanies the passage of established cell lines for months or years in vitro and/or in vivo.

A lengthy list of potential factors can be cited to explain why cells removed from their microenvironment in vivo will undergo phenotypic alterations (24). These include, but are not limited to, the following: growth in

two-dimensional monolayer culture versus organized histotypic assemblies; loss of cell-to-cell communication; disruption of epithelial-mesenchymal cellular interactions; elimination of the complex patterns of autocrine, paracrine, and endocrine regulation; and selection for cells with rapid and/or unlimited proliferative capacities. The inevitable outcome of such perturbations is that the cell populations that are selected during serial cultivation in vitro may represent subpopulations of cells that exhibit what might be called a "basal phenotype" for the original cell type from which they are derived (14). The "basal phenotype" represents the assembly of constitutive biochemical functions that are compatible with sustained proliferation and survival in an aberrant microenvironment devoid of the multiple inductive stimuli needed to maintain the more complex differentiated phenotype of the parent cell in its original location in vivo. The "basal phenotype" may include continued expression of a number of phenotypic traits unique to the cell of origin, including such traits as tumorigenicity and metastatic ability. However, other phenotypic markers, including some which may be of great importance in determining cellular refractoriness or the susceptibility of tumor cells to therapy, may be lost at variable rates following removal from the in vivo microenvironment.

The technical demands of examining these questions are formidable. A fundamental prerequisite will be to develop a panel of reliable phenotypic markers which can be used to compare human and animal tumor cells isolated from tumors of comparable histologic origins at similar stages in their progression to monitor possible changes in the expression of specific markers in tumor cells during passage ex vivo. Since cultured tumor cell populations will continue to be used as a major research tool for the foreseeable future, it will be equally important to establish the nature and the rate of phenotypic "drift" in cells harvested from tumors. This will require comparison of specific markers in tumor cells in vivo, in freshly isolated primary cultures, in low-passage secondary cell strains, and in established cell lines subjected to prolonged passage in vitro and in vivo. Only by undertaking this demanding comparison can we gain insight into the extent of phenotypic modulation that may occur in established cell lines used routinely in studying the mechanism of metastasis and in screening for new antineoplastic agents. Without this information, we will have little guarantee that current experimental strategies are focusing on tumor cell properties that are relevant to the goal of detecting antineoplastic agents with improved clinical efficacy against target neoplasms of defined histologic origin.

## Molecular Biology of the Malignant Tumor Cell and the Search for New Therapeutic Targets

The outlook for dramatic advances in the chemotherapy of the major human malignancies over the next 5 years is gloomy. Efforts to achieve improved clinical results will focus on the use of more aggressive adjuvant therapy regimens, the use of combinations of agents and/or modalities regimens, and more sophisticated staging of the use of individual agents in combination therapy to limit opportunities for the emergence of resistant cell variants (10). Due to limitations in current antineoplastic agents, these strategies will probably at best yield only small gains in patient survival statistics. Most of the new investigational cytotoxic agents in current clinical trials that could be approvable within 5 years resemble existing agents in their mode of action, and there is no a priori reason to assume that they will produce any quantum improvements in therapy. Deliberate drug design efforts to limit the toxicity of new agents may be of considerable benefit, however, in improving the quality of life for cancer patients. The major limitation is that current drug classes are inadequate for effective therapy of many tumors. Agents with novel mechanisms of actions are needed urgently for the treatment of tumors that are as relatively refractory or refractory to current therapies. Other than the serendipitous discovery of such agents via random screening, rational design of novel antineoplastic agents will require identification of new therapeutic targets in tumor cells and parallel progress in developing strategies to circumvent tumor cell heterogeneity and to achieve greater selectivity in killing tumor cells either via direct drug action or targeted drug delivery. Fulfillment of these ambitious objectives will require a far more sophisticated understanding of the molecular biology of human cancer cells and the mechanisms that regulate phenotypic diversification in specific tumor cell lineages. Against this rather depressing backdrop, it is hardly surprising that recent developments in molecular genetics, biotechnology, and immunology have been acclaimed by both the scientific and lay press as heralding a new era in our understanding of cancer along with the implicit (and in some cases, overly explicit) expectation that a "cure" will undoubtedly and quickly follow. Notwithstanding the premature nature of such claims (56), a consensus is emerging that the successful blending of molecular and cancer biology will inspire new research efforts predicated upon a molecular understanding of tumorigenesis and metastasis, and provide a welcome intellectual relief from the random and semiempirical approaches that have been the hallmark of past and present antineoplastic drug discovery programs. However, the transfer of cancer research from foundations of empiricism to those of rationality does not guarantee expeditious curtailment of malignant neoplastic disease as a major clinical problem. Many formidable hurdles remain to be surmounted and none more important than understanding the biochemistry of metastasis. As recognized throughout the clinical literature, the major problem in the treatment of cancer is the eradication of metastases, yet the amount of research devoted to elucidating the pathogenesis of metastasis and identifying the tumor cell properties re-

quired for metastatic dissemination accounts for a surprisingly small fraction of the current effort in basic cancer research. Consequently, the molecular processes underlying the expression of malignant behavior remain as elusive as ever. For example, the last few years have witnessed a sustained and rewarding interest in the role of oncogenes in tumorigenicity (57-59), but the participation of these entities in the metastatic process is obscure and has received far less attention (60-63). The evidence that aberrant oncogenes and/or overproduction of particular oncogene products can contribute to the expression of cellular immortalization, morphological transformation in vitro, and the formation of benign tumors in vivo is compelling. However, the question of whether the same (or different) oncogenes are essential, or even necessary, for the spread of tumor cells to distant organs and the proliferation of micrometastases remains unestablished. Answers to these questions will be forthcoming over the next few years and there is little doubt that they will have profound implications for developing future therapies for malignant neoplasms.

Notwithstanding the precise outcome of these questions, the application of molecular genetics to cancer biology has already influenced metastasis research. Molecular genetics offers an exciting new dimension for the analysis of the molecular correlates of malignant behavior and the identification of new therapeutic targets. Although the detailed phenotyping of malignant cells may be viewed by many as an oversimplistic interrogation of a multifactorial pathological process such as metastasis, it represents the first step in attempting to understand the malignant phenotype in terms of genes, gene expression, and gene products. Backed by the formidable analytical capacity of modern molecular biological techniques, we can perhaps reasonably expect to witness over the next few years progress in understanding the genetic, epigenetic, and regulatory mechanisms that underlie, contribute to, or influence the metastatic process and which might be exploited as potential routes for future pharmacological interventions.

The value of molecular biological techniques in searching for properties that endow tumor cells with "dangerous" characteristics such as metastatic abilities and resistance to diverse therapeutic agents will not be realized, however, unless experimental systems are used which embrace current concepts of tumor cell biology and tumor cell heterogeneity in particular. As discussed at length elsewhere (1,2,6,15,24), the coexistence within the same tumor (or tumor cell line) of subpopulations of cells with different metastatic properties dictates that the use of heterogeneous populations containing both nonmetastatic and metastatic cells cannot be interpreted as yielding information about the metastatic subpopulations. If, for example, metastatic cell subpopulations are in the minority, analysis of the entire cell population may provide little or no insight into the properties that are unique to metastatic cells, since the probability is high that these would be obscured or lost in the "background noise" imposed by the majority fraction of nonmetastatic cells. Consequently, characterization of the properties of metastatic tumor cell subpopulations requires that such subpopulations either comprise the overwhelming majority in a heterogeneous cell population or that cloning is used to generate homogeneous cell preparations in which metastatic and nonmetastatic clones isolated from the same parent cell population can be compared. In comparing the phenotypes of metastatic and nonmetastatic clones isolated from the same parent cell population, it is necessary, however, to confirm that the metastatic properties of the clones are stable. This imposes the need for repeated subcloning at intervals of only a few weeks to examine whether the metastatic behavior of the original clone has been maintained or if variant subclones with significantly different metastatic properties from the original clone have emerged to recreate a heterogeneous cell population. For clones showing rapid drift in metastatic behavior (or other properties), correlation of a specific cell property assayed in vitro with metastatic capacity in vivo requires that the in vitro and in vivo assays are conducted synchronously using replicate cell preparations (24,64). Unless such precautions are taken, a property assayed in vitro today cannot be correlated with the metastatic behavior of the same "clone" measured a few weeks earlier or later, because rapid formation of variant subclones will render the "clone" heterogeneous. Finally, reliable correlation of a specific phenotypic trait assayed in vitro with metastatic performance in vivo will require confirmation that the same trait is expressed by metastatic cells in vivo and also in cells recovered from metastatic lesions and recultured in vitro.

The demands of these more stringent experimental protocols are substantial. The proposals discussed earlier concerning the need for a greater emphasis on research on human tumor cells that share demonstrable phenotypic relatedness to tumor cells in situ are pertinent in this context and introduce additional technical complexities. In our opinion, however, unless attention is given to these issues, the search for tumor cell properties that correlate with clinically important phenotypes such as metastasis and therapeutic responses will have a low probability of success. Failure to address these problems over the past decade has dictated that an extremely large body of research using the newest techniques in cell biology, immunology, and biochemistry has been conducted with little regard for the pathogenesis of cancer, and metastasis in particular. This cycle could all too easily be repeated in the understandable desire to apply the next generation of emerging techniques in molecular biology to the study of cancer.

## CONCLUSION

If the answers to the questions posed in this article were easy, they would have been identified long ago. The

difficulties of discarding familiar and established experimental systems and retooling to test new models and paradigms that reflect advances in our understanding of tumor cell biology, immunology, genetics, and the pathogenesis of different cancers are not easy. The cost in time, effort, and resources will make many reluctant even to contemplate the need for change, yet alone implement it. Also, short-term cycles of grant funding, the pressure to maintain active publication records, bureaucratic inertia, and, for industrial laboratories, the need to continue to generate new drugs, add further barriers to radical shifts in experimental strategies. For those intrepid enough to begin to discard some, or all, of the traditional approaches, success will not be rapid. Worse still, there is no guarantee that adoption of some of the approaches described in this article will be any more successful than the methods they replace. We cannot, however, allow such fears to paralyze efforts to adopt new systems that embrace advances in our understanding of the pathogenesis of cancer metastasis. The choice is clear. The search for new therapeutic agents to combat metastatic disease must either continue using tumor models and experimental strategies that have not, and probably cannot, yield the desired outcome or we must embark upon the slow process of developing new strategies that attempt to integrate newer knowledge about the biology of the tumor(s) of interest. A decision to maintain the status quo, particularly in the search for chemotherapeutic agents that act in the same way as existing agents and which are identified and evaluated using nonmetastatic tumor models, carries the risk that in 10–20 years our success in treating the major solid tumors of man will be no greater than today.

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# Induction of Tumor Cell Differentiation as a Therapeutic Approach: Preclinical Models for Hematopoietic and Solid Neoplasms[1]

**Michael Reiss, Christina Gamba-Vitalo, and Alan C. Sartorelli[2,*]**

The majority of drugs available for anticancer therapy were designed to exert their action through the production of cytotoxic lesions. Although significant advances have been achieved with these cytodestructive agents in a number of malignant diseases, particularly those of childhood, this response is often attained at the expense of significant morbidity, and the clinical course of the most common malignant tumors in adults is, for the most part, only moderately improved by currently available cytotoxic regimens (1). Thus, for example, the successful treatment of childhood lymphocytic leukemia by cytotoxic agents has not been paralleled by comparable advances in the treatment of acute myelocytic leukemia (AML) of both children and adults. Since the late 1960s, the use of cytotoxic agents has produced remission in 50% of patients treated for AML (2); however, a high proportion of patients with AML fail to obtain benefit from current therapy, and the duration of survival in responders usually does not exceed 1.5–2 years (3,4). Moreover, it appears that the rate at which advances in the treatment of cancer are being made is leveling off.

Since the concept of tumor cell kill does not appear to be a sufficient long-range approach to cancer therapy, the development of new kinds of cancer chemotherapeutic agents whose mechanisms of action do not involve cytodestruction is sorely needed. One such approach, based on an understanding of the pathophysiology of malignancy, envisions the use of pharmacological agents as inducers of tumor cell maturation as a new form of cancer treatment (5–7). Experimental evidence accumulated over the past 15 years has demonstrated that tumor stem cells, both in hematopoietic and solid malignancies, give rise to differentiated end-stage cells at a rate significantly lower than that of corresponding normal tissues (6,8–10). Chemical induction[3] of differentiation[4] in vitro of malignant tumor cells of germ cell origin, as well as of cells derived from each of the three germ layers, has been accomplished (tables 1–4), indicating that the malignant process is not an irreversible one. Furthermore, studies indicate that differentiation therapy can be applied successfully in vivo in animal systems (55,88–90,108–112). The attainment of effective candidate drugs for clinical trial which act through the induction of maturation requires the availability of suitable preclinical in vitro and in vivo model systems representative of the most common clinical tumor types. Furthermore, the development of systematic therapeutic strategies to treat malignant diseases such as AML would appear to require an understanding of the regulation of granulopoiesis at both the cellular and humoral levels. Several in vivo leukemic models which permit the measurement of cellular and humoral factors on leukemic cells are described that also appear to be useful test systems to evaluate the efficacy of differentiating agents. The concept of solid tumor differentiation is discussed in terms of the potential of this approach for therapeutic gain, and experimental systems are described in which chemical induction of differentiation has been shown to occur, describing the types of drugs that have been utilized to accomplish this end.

## Growth and Differentiation in Malignant Tissue

Embryonic development is characterized by the initial proliferation of totipotent germ cells followed by a gradual and orderly differentiation of the tissues that form the adult organism (113,114). In certain tissues, such as bone marrow, testes, skin, and other living epithelia, fully differentiated mature cells are continuously lost and replaced by a proliferating pool of self-renewing stem cells during most of the life of the adult organism (115). These self-renewing tissue stem cells have a restricted competence[5]; that is, they are capable of giving rise to only one type (eg, in epidermis) or at most a small number of types (eg, in bone marrow) of mature progeny.

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[2]Departments of Medicine and Pharmacology, and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, CT.

[3]Process whereby external or internal regulators lead to the commitment of a stem cell to enter a certain maturation pathway.

[4]Process whereby the ability of a stem cell to give rise to multiple types of mature cells with specific functions gradually becomes restricted to one pathway of development.

[5]Inherent capability of a stem cell to give rise to one (unipotent), multiple (multipotent), or any (totipotent) type of mature cell.

*_Reprint requests to:_ Dr. Alan C. Sartorelli, Department of Pharmacology, Yale University School of Medicine, Sterling Hall of Medicine, PO Box 3333, New Haven, CT 06510-8066.

Table 1. Chemical Induction of Leukemia Cell Differentiation.

| Cell line | Treatment | Concentration | Parameters | References |
|---|---|---|---|---|
| HL-60 | DMSO[1] | 1.8-2.1 x 10$^5$ µM | increased $O_2^{-}$[2], increased HMP shunt[3], morphology, phagocytosis | 11-13 |
| | TPA[4] | 0.016 µM | NBT[5], morphology, surface receptors, $O_2^{-}$, HMP shunt, enzymatic characteristics | 14-17 |
| | 1,25-dihydroxy vit $D_3$ | 0.010-10.0 µM | morphology, enzymatic characteristics, lysozyme production | 17,18 |
| | RA[6] | 1-10 µM | surface antigens | 19,20 |
| | 2-β-D-Ribofuranosylselenazole 4-carboximide | 0.010 µM | morphology, NBT, functional characteristics | 21 |
| | Hypoxanthine | 5 mM | morphological, functional | 22 |
| | Dactinomycin | 0.004 µM | morphological, functional | 22 |
| | Aclarubicin | 0.03 µM | biochemical, functional, decreased glycoprotein synthesis | 23,24 |
| | Marcellomycin | 0.04 µM | biochemical, functional, decreased glycoprotein synthesis | 23,24 |
| | Tunicamycin | 0.6 µM | Fc[8], morphology, phagocytosis | 24,25 |
| | CSF[7] | | clonogenicity | 26 |
| | DF[9] | | NBT, morphology | 27-29 |
| KGl | TPA | 0.1 µM | morphology, histochemical, NBT, receptors | 30 |
| | Dexamethasone | 10 µM | colony formation | 31 |
| MLl and ML3 | TPA | 0.1 µM | morphology, histochemical NBT, receptors | 30 |
| | Ara-C[10] | 20 µM | receptors, cell cycle analysis, NBT, acid phosphatase | 30 |
| Ml | Tunicamycin | 0.6 µM | Fc, phagocytosis, morphology | 25 |
| | Glucocorticoid hormones | 2-50 µM | morphology, phagocytosis | 32 |
| | 1,25-dihydroxy vit $D_3$ | 0.001-0.012 µM | Fc, $C_3$, phagocytosis | 33 |
| | DMSO | 14 µM | Fc, $C_3$, phagocytosis, lysozyme | 34 |
| | Dactinomycin | 0.004 µM | EA receptors[12] | 35 |
| | Ara-C | 0.4 µM | EA receptors | 35 |
| | 5-IdUR[13] | 1.4 µM | EA receptors | 35 |
| | RA | 10 µM | lysozyme acid protease, acid phosphatase | 36 |
| WEHI-3BD$^+$ | RA | 0.5-1 µM | clonogenicity, Fc, morphology, NBT | 37,38 |
| | Aclarubicin | 0.03 µM | clonogenicity, Fc, morphology, NBT | 38 |
| | Doxorubicin | 0.04 µM | clonogenicity, Fc, morphology, NBT | 38 |
| | Carminomycin | 0.015 µM | NBT | 38 |
| K562 | 5-Azacytidine | 25 µM | globin synthesis | 39 |
| | Hemin | 40-50 µM | Hb[14] synthesis, globin m-RNA, structural protein | 40-42 |
| | Butyric acid | 1400 µM | Hb synthesis | 42 |
| | Doxorubicin | 0.04 µM | Hb and non-Hb proteins | 42,43 |
| | Dactinomycin | 0.5 nM | Hb synthesis | 44 |
| Friend | Ara-C | 0.7 µM | Hb synthesis | 45 |
| | Mitomycin C | 0.3 µM | Hb synthesis | 45 |
| | 6-Thioguanine | | Hb synthesis | 46 |
| | HMBA[13] | 5 mM | clonogenicity, Hb synthesis | 47,48 |
| | Butylated hydroxytoluene | 0.36 µM | Hb synthesis | 49 |
| | DMSO | 280 mM | Hb synthesis | 50,51 |
| U-937 | DF[10] | | morphology, biochemical | 52 |

[1]DMSO, dimethylsulfoxide, [2]$O_2^{-}$, superoxide formation; [3]HMP, hexose monophosphate shunt; [4]TPA, 12-O-tetradecanoyl-13-phorbol-12-acetate; [5]NBT, nitroblue tetrazolium; [6]RA, retinoic acid; [7]CSF, colony stimulating factor; [8]Fc, IgG receptor; [9]DF, differentiating factor; [10]Ara-C, cytarabine; [11]EA receptors, antibody coated erythrocyte; [12]5-IdUR, 5'-Iodo 2'-deoxyuridine; [13]Hb, hemoglobin; [14]HMBA, hexamethyl(bis)acetamide.

Table 2. Chemical Induction of Murine Embryonal Carcinoma Cell Differentiation.

A. IN VITRO

| Cell line | Treatment | Concentration | Parameters | References |
|---|---|---|---|---|
| F9 | RA[1] | 10 nm–1 μM | morphology, plasminogen activator | 53 |
| | RA + DBcAMP[2] | 0.1 μM+1 mM | morphology, plasminogen activator, alkaline phosphatase, antigen | 54 |
| | RA derivatives | 0.1–10 μM | morphology, plasminogen activator, α-fetoprotein | 55 |
| | Retinol | 1 μM | morphology, cell surface antigens, plasminogen activator | 56 |
| | Retinoidal benzoic acid derivatives | 1 nM–1 μM | plasminogen activator | 57 |
| | 5-BdUR[3] | 0.15 mM | plasminogen activator | 58 |
| | Hydroxyurea | 1 mM | plasminogen activator | 58 |
| | Ara-C[4] | 1.8 μM | plasminogen activator | 58 |
| | Mitomycin C | 12 μM | plasminogen activator | 58 |
| PCC4-ara 1 | Retinol | 1 μM | morphology, cell surface antigens, plasminogen activator | 56 |
| | DMA[5] | 0.1 mM | morphology, alkaline phosphatase, cell surface antigens | 59 |
| | HMBA[6] | 2 mM | morphology, alkaline phosphatase, cell surface antigens | 59 |
| | Polybrene | 8 μg/ml | morphology, alkaline phosphatase, cell surface antigens | 59 |
| PCC3 A/L | HMBA[6] | 5 mM | morphology, loss of clonogenicity | 60 |
| OC 15S | Retinol | 1 μM | morphology, cell surface antigens, plasminogen activator | 56 |

B. IN VIVO

| Tumor | Host | Treatment | Dose | Parameters | References |
|---|---|---|---|---|---|
| F9 | 129/SV mouse | RA | 50–100 mg per day orally | tumorigenicity, survival, tumor histology, plasminogen activator | 55 |
| PCC4-aza-1 | 129 mouse | RA in DMA | 200 μg in 20 μl DMA per day x 10 intratumor | survival, tumor histology | 61 |

[1]RA, retinoic acid; [2]DBcAMP, dibutyryl cyclic AMP; [3]5-BdUR, 5-bromodeoxyuridine; [4]Ara-C, cytarabine; [5]DMA, N,N-dimethylacetamide; [6]HMBA, hexamethylenebisacetamide.

In the bone marrow, the myeloid cell compartment is composed of three types of cells which differ in their functional capacities. The first compartment is composed of pluripotent stem cells which are capable of self-renewal or differentiation into more mature cells. The second contains cells committed[6] to the granulopoietic pathway; cells in this compartment are lineage specific, have limited self-renewal potential, are tightly associated with the differentiation of stem cells, and are responsive to regulation by humoral factors. Mature end-stage cells (ie, bands and polymorphonuclear cells) are located in the third compartment; these cells lack proliferative potential and are essential for specialized functions within the body. The self-renewal capacity of cells in the first two compartments allows stem cells to maintain independent clones. The differentiation of cells leads to mature elements with no self-renewal capability, but which are functionally effective for a finite life span. Under "steady-state" hematopoiesis, the level of cellular elements within each compartment works in tandem to regulate transition, level of cellular elements, and egress through each compartment. This regulation is also dependent on the levels of humoral factors such as colony-stimulating factor (CSF) and differentiating factors (DF). In addition to the above factors which regulate the rate of proliferation and differentiation, stochastic and deterministic mechanisms also contribute to the regulation of hematopoiesis. Models of stochastic probability imply that birth and death processes are random events in

[6]Restriction of the development of a stem cell to one pathway of maturation with subsequent loss of self-renewal capability.

Table 3. Chemical Induction of Neuroblastoma Cell Differentiation.

A. IN VITRO

| Cell line | Treatment | Concentration | Parameters | | | References |
|---|---|---|---|---|---|---|
| | | | Growth inhibition | Morphology | Function | |
| C1300[1] | DBcAMP[2] | 0.5 mM | + | + | + | 62-64 |
| | Papaverine | 70 µM | + | + | ND[3] | 65 |
| | RO7-2956[4] | 0.8 mM | + | + | ND | 65 |
| | RO20-1724[5] | 0.7 mM | + | + | ND | 65 |
| | Prostaglandin $E_1$ | 30 µM | + | + | ND | 66,67 |
| | Prostaglandin $E_2$ | 30 µM | + | + | ND | 66,67 |
| | Prostaglandin $A_2$ | 40 µM | + | + | ND | 68 |
| | Sodium butyrate | 0.5 mM | + | - | + | 69 |
| | FdUR[6] | 10-100 µM | + | + | ND | 70,71 |
| | BdUR[7] | 1 µM | ND | + | ND | 72,73 |
| | 6-Thioguanine | 0.5 µM | + | + | + | 72 |
| | Ara-C[8] | 3 µM | + | + | + | 71,74 |
| | Methotrexate | 2.0 mM | ND | + | ND | 74 |
| | DTIC[9] | 60 µM | + | + | + | 75 |
| | Doxorubicin | 2 pM-0.75 mM | + | + | + | 76,77 |
| | Bleomycin | 0.002 U/ml | + | + | + | 77 |
| | DMSO[10] | 140-280 mM | + | + | + | 78 |
| | HMBA[11] | 2-5 mM | + | + | + | 79 |
| | α-Tocopherol | 0.05 IU/ml | + | + | ND | 78,80 |
| | Dexamethasone | 30 µM | + | + | + | 81 |
| | Valinomycin | 10 nM-10 µM | + | + | + | 82,83 |
| | Nerve growth factor | 4 ng/ml | + | + | + | 84 |
| IMR-32[12] | Papaverine | 7 µM | + | + | + | 85 |
| | RO20-1724 | 0.7 mM | + | + | + | 85 |
| | Prostaglandin $E_1$ | 30 µM | + | + | + | 85 |
| | Sodium butyrate | 0.5 mM | + | + | + | 85 |
| | RA[13] | 1-10 µM | + | + | + | 86 |
| LA-N-1[12] | RA | 1-10 µM | + | + | + | 86 |
| LA-N-2[12] | RA | 1-10 µM | + | + | + | 86 |
| KA[12] | RA | 1-10 µm | + | + | + | 86 |
| SK-N-SH[12] | RA | 1-10 µM | + | + | + | 86 |
| CHP-134[12] | RA | 1-10 µM | + | + | + | 86 |
| SH-SY 5Y[12] | TPA[14] | 16 mM | + | + | + | 87 |

B. IN VIVO

| Tumor | Host | Treatment | Dose | Parameters | References |
|---|---|---|---|---|---|
| MNB-$P_2$[15] | A/J mouse | DBcAMP and papaverine and theophylline | 1 mg/kg ip x 7<br>7.5 mg/kg ip x 7<br>15 mg/kg po x 7 | tumor growth | 88 |
| | mouse | Prostaglandin $E_1$ and RO20-1724 | 100 µg intratumor<br>200 µg intratumor | tumor regression | 89 |
| Human | nu-mouse | Papaverine | | tumor growth | 90 |
| Human neuroblastoma | patients | Papaverine | 45 mg/kg iv 3-4 weeks and cytoxan. VCR[16],$F_3$TdR[17] | 14/15 responses | 90,91 |

[1]C1300, different sublines of the murine neuroblastoma C1300 were used in various reports; [2]DBcAMP, dibutyryl cyclic AMP; [3]ND, not determined; [4]RO7-2956, 4-(3,4-dimethoxybenzyl)-2-imidazolidinone; [5]RO20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; [6]FdUR, 5-fluorodeoxyuridine; [7]BdUR, 5-bromodeoxyuridine; [9]Ara-C, cytarabine; [9]DTIC, 5-(3,3-dimethyl-1-triazenoimidazole-4-carboxamide; [10]DMSO, dimethylsulfoxide; [11]HMBA, hexamethylene bisacetamide; [12]IMR-32, LA-N-1, LA-N-2, KA, SK-N-SH, CHP-134, human neuroblastoma cell lines; [13]RA, retinoic acid; [14]TPA, 12-O-tetradecanoyl-13-phorbol-12-acetate; [15]MNB-$P_2$, murine neuroblastoma cell line; [16]VCR, vincristine; [17]$F_3$TdR, trifluoromethyl-2'-deoxyuridine.

Table 4. Chemical Induction of Epithelial Tumor Cell Differentiation.

| Cell line | Treatment | Concentration | Parameters | References |
|---|---|---|---|---|
| MELANOMA | | | | |
| S-91[1] | Choleratoxin | 0.1 nM | morphology, tyrosinase, melanin, DNA synthesis, adenylate cyclase | 92 |
| | MSH[2] | 10 nM | morphology, tyrosinase, melanin, DNA synthesis, adenylate cyclase | 92 |
| | RA[3] | 10 μM | morphology, melanin, cell growth | 93 |
| B-16[1] | Cyclic AMP | 3 mM | morphology, melanin, cell growth | 94 |
| | DBcAMP[4] | 1 mM | morphology, melanin, cell growth | 94 |
| | Caffeine | 1 mM | morphology, melanin, cell growth | 94 |
| | Theophylline | 1 mM | morphology, melanin, cell growth | 94 |
| B-16 F10[1] | Doxorubicin | 2 nM | morphology, cell growth | 95 |
| | N-Trifluoroacetyl-adriamycin 14-valerate | 10 μM | morphology, cell growth | 95 |
| HO[5] | DMSO[6] | 70-280 mM | morphology, melanin, cell growth | 96 |
| | TPA[7] | 0.5 nM-50 μM | morphology, melanin, cell growth | 96 |
| ADENOCARCINOMA-BREAST | | | | |
| RAMA[8] | DMSO | 280 mM | dome formation, casein secretion | 97,98 |
| | Prostaglandin $E_1$ | 15-150 μM | dome formation, casein secretion | 98 |
| | RA | 40 nM-3 μM | dome formation, casein secretion, DNA synthesis, tumorigenicity | 99 |
| ADENOCARCINOMA-COLON | | | | |
| DLD-1[9] | DMF[10] | 0.8% | tumorigenicity, cell surface antigens | 100,101 |
| HCT-15[9] | DMF | 0.8% | tumorigenicity, cell surface antigens | 100,101 |
| SW-480[9] | Sodium butyrate | 5 mM | cell growth, morphology, glycolipids | 102 |
| | DMSO | 280 mM | cell growth, morphology, glycolipids | 102 |
| SW-620[9] | Sodium butyrate | 5 mM | cell growth, morphology, glycolipids | 102 |
| | DMSO | 280 mM | cell growth, morphology, glycolipids | 102 |
| HT-29[9] | Sodium butyrate | 5 mM | clonogenicity, mucus production, dome formation | 103,104 |
| SQUAMOUS CELL CARCINOMA | | | | |
| SSC-4[11], SSC-9, SSC-12, SSC-13, SSC-15, SSC-25 | Semisolid medium | | clonogenicity, keratinization, cornified cell envelopes | 105 |
| SSC-13 | Hydrocortisone | 1 μM | clonogenicity, cornified cell envelopes | 106 |
| SqCC/Y1[11] | Serum deprivation | | stratification, keratinization, cornified cell envelope, clonogenicity | 107 |

[1]S-91, B-16, B-16 F10, murine melanomas; [2]MSH, melanocyte stimulating hormone; [3]RA, retinoic acid; [4]DBcAMP, dibutyryl cyclic AMP; [5]HO, human melanoma; [6]DMSO, dimethylsulfoxide; [7]TPA, 12-O-tetradecanoylphorbol-13-acetate; [8]RAMA, rat tumor; [9]DLD-1, HCT-15, SW-480, SW-620, HT-29, human colon carcinoma; [10]DMF, N,N-dimethylformamide; [11]SSC, SqCC/Y1, human head and neck carcinoma cell lines.

which some stem cells divide to give rise to additional stem cell progeny, whereas others yield more mature cellular elements (117). In contrast, deterministic mechanisms are not random events but rather have a direct cause and effect relationship, in which external events, such as the microenvironment, circulating humoral factors, or preprogramming, direct a stem cell to either proliferate or differentiate (118).

Carcinomas appear to arise almost exclusively in self-renewing tissues (8); the tissue stem cells are the most likely targets of the malignant transformation. In non-renewing tissues, such as neurons, the carcinogenic event most probably involves a process of dedifferentiation. In both instances, the malignant transformation produces an altered pattern of differentiation that results in a clone with unlimited proliferative capacity. A malignant tumor, however, is usually not composed exclusively of undifferentiated stem cells. Thus, carcinomas, for example, often contain abortive representations (eg, horny pearls, ductules) of the mature cells or structures which are ordinarily found in the corresponding nonneoplastic tissue (119). These morphological characteristics suggest that tumor stem cells retain the ability to give rise to differentiated progeny of the tissue of origin. Evidence that this is indeed the case has been provided by elegant experiments performed by Pierce and Wallace (120) using a transplantable squamous cell carcinoma (SqCC) of rat epidermis. Serial biopsies of tumors taken after in vivo pulse labeling with tritiated thymidine revealed that the radioactivity initially present in undifferentiated cells was transferred to the keratinized cells of the horny pearls through cellular maturation. Furthermore, keratinized cells transplanted to other rats were demonstrated to no longer be tumorigenic. These experiments indicated that a transition took place in vivo within the mass of replicating tumorigenic cells to form morphologically mature, nonmalignant progeny. A second dramatic example of the evolution of a tumor stem cell to a mature, nonmalignant end-stage progeny was provided by Illmensee and Mintz (121), who demonstrated that single murine embryonal carcinoma cells injected into early-stage mouse embryos (ie, blastocysts) gave rise to mature tissues which formed an integral part of normal chimeric mice that arose from these embryos.

Several lines of evidence exist which indicate that the regulation of differentiation is disturbed in malignancy, leading to a decrease in the rate of cellular differentiation relative to proliferation. Examples of this evidence include the studies of Frankfurt (122), who used tritiated thymidine labeling to measure the fraction of differentiating cells in the normal squamous epithelium of the forestomach of mice compared to tumor tissue of the same site. He found that in the steady-state situation, approximately 50% of the cells of the normal mucosa became keratinized, whereas in carcinomas arising in the same tissue only 25% of the cells underwent maturation. More recently, Yuspa and Morgan (123) reported that the transformation induced by carcinogens in mouse skin was accompanied by the development of resistance to conditions that led to the terminal differentiation of normal keratinocytes. Similarly, Parkinson et al (124) found that SV-40-transformed epidermal cells no longer responded to the induction of differentiation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as nontransformed cells do. In addition, cells derived from human squamous cell cancers matured much more slowly and incompletely under culture conditions which led to rapid keratinization of normal skin cells (105). Similar observations have been reported for mesenchymal cells, in that neoplastic transformation of 3T3-proadipocytes resulted in a loss of the ability to undergo maturation to end-stage adipocytes (125).

In a perturbed situation such as leukemia, a dissociation of the regulatory events controlling the self-renewing and differentiating programs of stem cells also occurs (126). In addition, negative feedback regulation between the three compartments becomes inoperable (127,128). Hematopoietic dysplasias like chronic myeloblastic leukemia (CML), polycythemia vera, idiopathic myelofibrosis, and AML are considered to be clonal diseases, in that the heritable lesion is in the pluripotent stem cell compartment (129). These cells give rise to clones which ultimately become dominant and crowd out normal hematopoietic elements. In AML, an aberration at the pluripotent stem cell level can manifest itself as a block in differentiation at the promyelocyte stage of differentiation (129). These immature cells are unable to progress into morphologically and functionally mature granulocytes and rapidly undergo clonal expansion. That this aberration is clonal in nature and originates from a stem cell is documented in reports demonstrating the association of the Philadelphia chromosome with CML cells (130), and the presence of only one type of glucose-6-phosphate dehydrogenase isozyme in CML granulocytes, whereas the double enzymatic phenotype is present in skin and cultured fibroblasts from the same patient (131).

The change in the total mass of leukemic cells depends not only on the proportion of cells capable of proliferating (ie, the growth fraction), but also on the rate of proliferation and the proportion of cells which fail to proliferate (4). The growth fraction in a leukemic marrow is composed of populations of leukemic cells which are actively dividing or either resting or very slowly proliferating. The low mitotic indices observed for early leukemic blasts suggest that very few leukemic cells are actively dividing (132). Thus, the majority of leukemic cells are probably confined to a mitotic resting stage, ie, $G_0$ (133) or restricted to the $G_1$ phase (134) of the cell cycle. These cells are accompanied by a population of blast-like forms that are actively replicating.

Responses produced in clinical trials using retinoic acid (135,136), aclarubicin (137), dactinomycin (138), low-dose cytarabine (138), and sequential high-dose cytarabine

and asparaginase (139) in patients with acute nonlymphocytic leukemia have been suggested to be possibly due to the induction of differentiation. It is unclear, however, whether the outcome of such trials was due to the maturation of leukemic cells or to cytotoxicity to the neoplastic cells which lowered the tumor burden in a manner that allowed normal clones of stem cells to be reexpressed. For this reason, it is critical that future clinical trials devise strategies that permit a distinction to be made between these mechanisms; such strategies may well require appropriate methodology to be developed in in vivo animal model systems. The use of semisolid or liquid cultures of bone marrow cells from humans to assess the degree of maturation of leukemia cells is difficult to interpret (140). The possibility that normal hematopoietic cells are present in the marrow of these patients creates an unacceptable variable in interpreting the results of the action of chemotherapeutic agents on leukemic cells, since differentiated normal cells can arise in cultures treated with toxic agents, making the origin of mature cells inconclusive.

In summary, existing evidence supports the concept that malignant tumors arise in self-renewing tissue stem cells or their progeny which, in many if not in most instances, appear to have retained the ability to enter a pathway of terminal differentiation similar to that occurring in the nonneoplastic tissue from which the tumor arose. McKillop et al (141) have recently formulated a mathematical model for the growth kinetics of tumors based upon these premises. According to this model, tumor cells belong to one of three categories: (*a*) proliferating, self-renewing stem cells; (*b*) transitional cells committed to terminal differentiation, but with the potential to undergo a limited number of cell divisions; and (*c*) nonproliferating mature end-stage cells. The growth rate of a solid tumor thereby depends mainly upon three factors: (*a*) the probability that the mitotic division of a stem cell will yield two new stem cells rather than two progeny cells committed to maturation (ie, the $P_{SR}$, probability of self renewal); (*b*) the number of cell divisions (clonal expansion) a committed cell is able to undergo; and (*c*) the rate of cell loss. Assuming that the rate at which end-stage cells are removed through loss from the tumor is negligible, the probability of cellular self-renewal largely determines tumor growth rate. A continuous increase in the size of the stem cell compartment will occur if $P_{SR} > 0.5$, and the growth of the tumor will continue unabated. However, even in this situation (ie, $P_{SR} > 0.5$), the majority of tumor cells will consist of transitional or end-stage cells, a situation consistent with experimental evidence. At present, tumor development can only be reduced by surgical removal or by cell kill using radiation therapy or cytotoxic chemotherapy. However, if the $P_{SR}$ could be reduced to $< 0.5$, the growth rate of the tumor would gradually decrease. These cell kinetic considerations provide a rationale for the application of "differentiation therapy," ie, the use of drugs or other techniques that increase the likelihood that a dividing tumor stem cell will yield committed progeny rather than new stem cells. One can also infer from this model that the results of a combination of cytoreductive drugs with those capable of inducing differentiation might be superior to the application of either modality alone. Studies by Ferrero et al (142) with HL-60 acute promyelocytic leukemia cells support the use of such an approach.

## Model Systems for Measuring Tumor Cell Differentiation

That malignancy is not an irreversible phenomenon has been demonstrated with a variety of tumor types. Thus, teratocarcinomas (120,143), neuroblastomas (144,145), squamous cell carcinomas (120), leukemias (146–148), and adenocarcinomas of the breast (149) and colon (150) all have been shown to be able to yield mature cellular forms. It is conceivable that the spontaneous remission of cancer which occurs infrequently may be due at least in part to differentiation of malignant stem cells to end-stage cells. To identify therapeutic agents capable of initiating the commitment to differentiate in malignant tumors, preclinical models are needed in which the rates of tumor stem cell renewal and differentiation can be measured accurately, and in which cytotoxic effects can be distinguished from the induction of maturation. Changes in the relative sizes of the tumor stem cell pool and the combination of transitional and end-cell populations over time will reflect the probability of self-renewal, assuming the selective loss of end-stage cells is negligible (141). Although clonogenic assays are currently utilized to provide a measure of the stem cell pool, technical problems associated with these assays suggest that they underestimate the actual number of tumor stem cells. Since no other independent method of identification of true malignant stem cells is available, however, the magnitude of the error incurred in these assays cannot be ascertained. Therefore, the most important criterion for a successful preclinical model for malignant tumors is the availability of unequivocal phenotypic characteristics of the terminally differentiated cell type (table 5). Markers that specifically identify differentiated cells in the nonneoplastic tissue type from which the tumor arose must also be present in maturing tumor

TABLE 5.—Optimum requirements for experimental models for preclinical testing of differentiation-inducing agents

1. Well-defined and measurable characteristics of the mature cell (or tissue) phenotype not present in tumor stem cells.
2. The capacity for measurements of clonogenicity in vitro, as well as for tumorigenicity in vivo, to correlate decreases in tumor stem cells with the appearance of the mature phenotype.
3. In vitro models should have an in vivo counterpart in which parameters of maturation can be measured to permit the influence of drug pharmacokinetics, therapeutic index, and the effect of host factors on tumor maturation to be estimated.

cells. Ideally, the marker(s) would be totally absent from the stem cells, but appear as soon as the cell enters the pathway of terminal differentiation, ie, shortly after commitment. Conversely, use can be made of a characteristic of the stem cell state which disappears upon commitment. The markers of the differentiated phenotype could include specific structural glycoproteins, enzymes, or secretory products, as well as the formation of multicellular structures (eg, stratified squamous epithelium, droplets and domes, tubules, etc). Of major importance to clinical utility is that the appearance of the differentiated phenotype be accompanied by a concomitant loss of proliferative potential. This will be reflected in a decrease in the size of the stem cell pool and thus of clonogenicity. Finally, preclinical testing of agents capable of inducing differentiation requires the availability of animal models with transplantable tumors that permit measurement of the influence of pharmacological parameters on the rate of tumor cell maturation induced by potential agents, as well as their therapeutic indices.

The first observation that neoplastic stem cells could be induced chemically to progress to nontumorigenic mature cells was reported approximately 15 years ago, with the demonstration that the formation of mature colonies from murine myelomonocytic leukemia cells could be enhanced by CSF (147). This finding was followed by reports that similar effects could be produced by human spleen conditioned medium on human leukemic myeloblasts (148), and by the polar solvent DMSO on Friend murine erythroleukemia cells (50). In an analogous manner, the induction of a fibroblast-like morphology and restoration of density dependent growth by dibutyryl cyclic AMP in Chinese hamster ovary cells was described (151), and neuroblastoma cells were shown to display mature characteristics when culture conditions were altered (145) or cells were treated with 5-bromodeoxyuridine (73). Since these original observations, a number of tumor cell systems of hematopoietic, epithelial, neural, and mesenchymal, as well as of germ cell origin, have been studied in detail. The ability to differentiate neoplastic cells as a therapeutic approach should ideally occur at a drug dose below the level at which toxicity is expressed, and the agent used should not cause the maturation of normal stem cells. Thus, the total tumor burden will be susceptible to reduction without major side effects on normal stem cells.

Since the late 1960s, a number of human and murine leukemic cell lines have been developed that have been used to study differentiation. These cell lines, which are composed of homogeneous neoplastic populations, allow some distinction to be made between the differentiating and cytotoxic actions of chemotherapeutic agents. Murine myeloid leukemic cell lines, such as the M1 (152) and the R453 (153), can be induced to differentiate in vitro into either macrophage- or granulocyte-like cells by treatment with agents such as endotoxin sera (154), dexamethasone (154), conditioned medium from spleen (152), peritoneal macrophages (153), and rat ascites proteinaceous inducer (154). The establishment of human myelogenous cell lines like HL-60 (155), KG1 (156), ML1 and ML3 (157), and U-937 (158) is also important to studies of leukemic cell differentiation, since these lines are blocked at different stages in the maturation process. KG1 cells are myeloblastic (156); HL-60 cells are composed mainly of promyelocytes (155); ML1 and ML3 lines are monomyeloblastic (157); and U-937 is a histiocytic lymphoma with monoblast-like characteristics (158). A variety of agents, such as polar-planar compounds (13), purine and pyrimidine analogs such as 3-deazauridine (159), a number of cancer chemotherapeutic agents (22–24), and vitamin analogs (18–20) are all initiators of HL-60 cell differentiation (table 1). In contrast, the KG1, ML1, and ML3 cell lines cannot be induced to mature by most of the above mentioned agents (160,161). The U-937 cell line can be initiated to differentiate to macrophage-like cells by agents such as TPA (162), retinoic acid (163), 1,25-dihydroxy vitamin $D_3$ (164), and a soluble factor produced by lectin-stimulated T lymphocytes (164).

The most widely studied of all of the leukemia models is the HL-60 cell line, derived from a patient with promyelocytic leukemia (165). Although HL-60 cells initially required conditioned medium to grow (155), the line currently does not need either production of endogenous factors or the addition of exogenous factors to proliferate. The morphological and histochemical characteristics of the cell line have remained similar to the characteristics of the leukemic cells obtained from the marrow of the patient prior to chemotherapy (165). Cytogenic studies have shown that initially these cells were aneuploid, with 44 chromosomes in the early passages of the line; however, with continued passage, a shift in the modal chromosome number to 45 or 46 has occurred. Since HL-60 cells can differentiate into granulocyte- or macrophage-like cells, it is an ideal system to employ to investigate the biochemical mechanisms that regulate the decision to enter different pathways of maturation. The criteria used to monitor the differentiation process should include a measurement of the cessation or reduction of cellular proliferation concomitant with morphological and biochemical changes, such as the generation of superoxide, an increase in the hexose-monophosphate shunt activity, the capacity for phagocytosis, motility, and specific enzyme content such as peroxidase, loss of ASD chloroacetate esterase, changes in Sudan black B staining, and increases in Fc and C3 receptors on the cell surface.

Although the use of cell lines in culture has simplified studies of leukemia cell differentiation, the absence of a biological microenvironment and circulating humoral factors makes interpretation of results with differentiating agents incomplete. Studies utilizing long-term marrow cultures, which approximate the normal milieu, suggest that stromal cells exert a local and necessary in-

fluence on hematopoiesis (166). This adherent layer, comprised of endothelial cells, reticular cells and adipocytes, is usually associated with macrophages (167). Studies using both chemotherapeutic agents (168,169) and irradiation (169,170) indicate that this layer is necessary for normal granulopoiesis. The possibility exists that relatively low doses of agents capable of inducing differentiation with minimal cytotoxicity would not severely perturb stromal elements and the normal production of factors by macrophages while inducing leukemic cells to terminate proliferation by their conversion to more mature end-stage cells. It is conceivable that (*a*) such mature elements would be capable of secreting chalones or "poietins" which would then regulate both the size of cellular compartments and the transit of cells through their respective domains, and (*b*) stromal elements could contribute directly to the induction process.

The role of humoral factors in inducing the differentiation of leukemic cells has been demonstrated both in vitro and in vivo (171,172). Thus, the injection of M1 cells into 10-day-old mouse embryos resulted in the differentiation of these cells to mature granulocytes (146). This finding indicates that embryonic factors are capable of inducing the maturation of M1 cells. Three groups of factors have been reported to be necessary for the development of normal granulocytes and macrophages: G-CSF, M-CSF, and GM-CSF. These CSFs are required at both the proliferation and differentiation stages of hematopoiesis. Therefore, under steady-state hematopoiesis it is difficult to separate the actions of CSF on the hematopoietic regulation of the processes of proliferation and differentiation. The use of myeloid leukemic cell lines, however, provides an opportunity to study the role of humoral factors in the regulation of these processes in malignant cells, since the formation of mature granulocytes and macrophages from myeloid precursors has been shown to be controlled both by CSF (173,174) and by DF (53,174,175). CSF has been reported to induce cell growth, whereas DF initiates cellular differentiation (52,173–175). MG1 leukemic cells incubated with agents like lipopolysaccharide (LPS) and tumor-promoting phorbol esters secrete CSF and DF-associated proteins into the medium (176–178). Similarly, the murine IR cell line has been induced to differentiate into granulocytes by conditioned medium derived from cultures of lungs from mice injected with endotoxin (179). The administration of endotoxin has been reported to stimulate the release of CSF from host macrophages (180). Murine models utilizing the phenomenon of drug-induced rebound myelopoiesis have demonstrated that the administration of transferrin and lactoferrin suppresses the normal cycling status of cells within the colony-forming unit-granulocyte macrophage (CFU-GM) compartment (181). These effects are probably also mediated by the production of factors necessary for the movement of cells into and through the CFU-GM compartment. HL-60 promyelocytic leukemia cells have been shown to be induced to differentiate to mature end-stage cells by a factor produced in cultures of stimulated mononuclear blood cells (27) and characterized as DF (29). WEHI-3BD$^+$ myelomonocytic leukemia cells produce a series of CSF and DF proteins which appear to be involved in the autoinduction of maturation (182). Thus, it is conceivable that in some leukemic models, chemotherapeutic agents may act to lower the tumor burden both through direct effects at the cellular level which induce differentiation and/or cytotoxicity as well as through indirect action by stimulating the production of humoral factors that initiate differentiation either from macrophages or from leukemic cells that have been converted to mature end-stage cells.

Because of the incompatibility of human leukemic cells with murine hosts, investigators using these lines have the option of employing either diffusion chambers or nude mice to study effects of differentiating agents in vivo; alternatively, several murine cell lines are available for investigations of this kind. Mouse M1 myeloid leukemia cells that have been placed into diffusion chambers in mice treated with LPS or dexamethasone differentiate into mature granulocytes (108,110). Using the same technique, HL-60 cells have been shown to differentiate in vivo into mature granulocytes when host mice were treated with the anthracyclines, marcellomycin, or aclarubicin (109). Although the diffusion chamber technique is useful for the in vivo analysis of compounds with the potential to cause maturation, this methodology precludes a study of drug action on the communication between the implanted leukemia cells and host normal hematopoietic cells and stroma, as well as of the effects of host humoral factors that modulate the maturation process. The nude mouse model has been used for the development of human myelosarcomas from KG1 and HL-60 leukemia cells (183). Although there is no evidence that immunocompetent cells are necessary for the differentiation of leukemia cells, under "steady-state" hematopoiesis, T-cells and macrophages have been associated with foci of erythropoiesis and granulopoiesis and humoral factor production (184,185). If these cellular elements are involved in modulating the differentiation of leukemic cells, then the nude mouse model would not be ideal for testing the potential of agents that act through inducing differentiation.

The murine MG1 cell line, derived from a spontaneous myeloid leukemia in the SL mouse (179), appears to be a reasonable murine model for testing potential differentiating agents in vivo. Both differentiation positive (D$^+$) and negative (D$^-$) sublines are available. The D$^+$ clone can be induced to differentiate along the granulocytic pathway by a variety of agents and can be stimulated to produce CSF. In contrast, the majority of D$^-$ clones cannot be induced to differentiate (179). Iv injection of MG1D$^+$ myeloid leukemia cells into SL or SJL/J mice causes myeloid leukemia with a median survival of 41 days (179). The administration of MG1-2 (DF) to tumor-bearing mice inhibited the in vivo development of leu-

kemia, whereas MG1-1 (CSF) had no effect (179). That this was due to a direct action on the leukemic cells is supported by results obtained in culture which demonstrate that MG1 cells treated with DF differentiate to granulocyte-like cells (186). Since clonal sublines exist that are either differentiation responsive or defective, MG1 cells would appear to be a useful model for the preclinical testing of potential differentiating agents. Since some of the sublines of MG1 are differentiation-defective and perhaps do not have receptors on their surface for the normal regulation by humoral factors, this model would be capable of assessing the interaction of differentiating agents alone as well as with circulating humoral factors. The appearance of surface receptors for humoral factors in response to differentiating agents may permit the assessment of the mode of regulation of maturation in this system.

Another potentially useful cell line for in vivo drug testing is the WEHI-3BD$^+$ monomyelocytic leukemia which was developed in the BALB/c mouse (187). We have preliminary data which indicate that the WEHI-3BD$^+$ cell line can be induced to differentiate into mature granulocytes when exposed to agents such as aclarubicin, carminomycin, doxorubicin, and retinoic acid in vitro (38). That these compounds terminate proliferation by inducing maturation rather than through the production of cytotoxicity is indicated by the de novo synthesis of Fc receptors. The finding of receptors for CSF on the surface of WEHI-3BD$^+$ cells is indicative of the similarities between this cell line and normal hematopoietic cells (188). Furthermore, secretion of hematopoietic factors by these cells has been shown to be necessary for both in vivo and in vitro cellular differentiation (189,190). Thus, this cell line is an excellent murine model for the in vitro study of potential therapeutic agents which induce differentiation by acting directly or through interaction with exogenous or endogenous hematopoietic humoral factors. Furthermore, the ability of WEHI-3BD$^+$ cells to produce a monomyelocytic leukemia involving host hematopoietic tissue when inoculated into BALB/c mice permits the in vivo investigation of (*a*) dose and scheduling of chemotherapeutic agents to minimize nonspecific cytotoxicity, (*b*) leukemic cell-host marrow microenvironment interactions, and (*c*) cell-cell interactions (leukemic cell-T-cell or macrophage interactions) that normally occur in leukemic patients.

The solid tumor type perhaps best known for the spontaneous occurrence of maturation or the induction of differentiation to benign tissue is the teratocarcinoma (191). These malignant tumors can arise either from germ cells in the testes, or from early embryos. Morphologically, the embryonal carcinoma stem cells (EC) are readily distinguishable from their differentiated progeny, which can consist of tissue derived from any one of the three embryonic germ layers: ectoderm (eg, epidermis, neural tissue), mesoderm (eg, muscle, bone, cartilage), or endoderm (eg, glandular structures, intestine). When tumors of this type are transplanted intraperitoneally into mice, they give rise to either simple or cystic embryoid bodies which are organized along the lines of early embryogenesis (191). Depending on the type of differentiated tissue formed, a variety of specific enzymes are induced [eg, plasminogen activator in parietal endoderm cells (192), creatine phosphokinase and acetylcholinesterase in muscle or nervous tissue (193)]; conversely, the transition from an EC to a differentiated cell is usually accompanied by the loss of alkaline phosphatase (194) and specific cell surface antigens (195). Apart from the spontaneous appearance of differentiated tissues within teratocarcinomas, it has been dramatically demonstrated by Illmensee and Mintz (121) that single murine EC stem cells injected into mouse blastocysts give rise to normal mature tissues which form an integral part of an entirely normal mouse chimera which arose from these blastocysts (121,143). Thus, the malignant behavior of EC stem cells can be completely reversed under appropriate environmental influences. A great number of clonal murine EC cell lines have been isolated with different capacities for growth and differentiation (191). Most of these cell lines are multipotent, ie, they spontaneously give rise to various types of differentiated tissue when cultured for prolonged periods of time, especially in serum-free medium (196). The extracellular environment appears to play a critical role in the regulation of this process, since the presence of a fibroblast-derived feeder layer (197) or medium conditioned by feeder cells (198) appears to inhibit differentiation. Conversely, the presence of fibronectin appears to enhance maturation (196).

In contrast to the multipotent EC cell lines, a small number of murine embryonal carcinoma lines have been isolated which do not differentiate spontaneously in vitro (199). However, in vitro maturation of these EC stem cells to one or multiple forms of differentiated tumor can be induced by chemical means which are summarized in table 2. Inducers of EC differentiation include physiological agents such as retinol and derivatives thereof, which are believed to act at the level of the genome (200); membrane-active nonpolar solvents (201); and inhibitors of DNA synthesis, which appear to exert their effects on cell differentiation at concentrations below those producing cytotoxicity (58). Changes in cell and tissue morphology, increases in enzymatic activity, and alterations in cell surface antigens are commonly used parameters of in vitro maturation (191-195). Loss of clonogenicity in vitro or tumorigenicity in vivo has not always been measured. Experimental results should be interpreted with caution, especially when inhibitors of DNA synthesis are employed, since the contribution of direct cytotoxicity with the concomitant selection of noncycling more mature cells is difficult to evaluate. However, retinoic acid treatment of EC-bearing mice decreased tumor growth by a mechanism that led to the appearance of relatively large fractions of differentiated cells in the tumors (55,61).

Although most of the observations with teratocarcinomas were obtained with murine cell lines, the establishment of several EC cell lines of human origin has also been reported recently (202,203). In contrast to most of the murine EC cell lines, those derived from human teratocarcinomas generally differentiate only poorly and incompletely in vitro (202,203). Moreover, attempts at initiating maturation with all-trans retinoic acid, as well as with a variety of other agents, in a number of these cell lines have not been successful (204). Thus, conclusions drawn from experiments performed with murine systems may not be directly extrapolatable to human teratocarcinoma.

In 1969, Klebe and Ruddle (70) reported that C1300 cells, a line derived from a mouse neuroblastoma, could undergo morphological differentiation in vitro. These cells are normally propagated in suspension culture; however, in the presence of a suitable substrate to which C1300 cells will attach, 10%–20% of the cells form long processes which have ultrastructural characteristics of mature neurites, including the "end-cones" typical of mature neurons (62,144). Moreover, the cell membrane acquires the ability to generate action potentials in response to electrical or chemical stimulation (82,83). In certain cloned lines derived from the C1300 tumor, the production of enzymes involved in the synthesis and turnover of neurotransmitters (eg, tyrosine hydroxylase and acetyl cholinesterase) can be stimulated when cells are induced to mature (205). Furthermore, the differentiated cells acquire neurotransmitter receptors on their surface (205). The appearance of mature cells in vitro appear to be affected by two factors also noted for EC cells. First, a suitable substrate must be present; thus, attachment appears to be critical for neuroblastoma cells to form neurites (144), and second, the composition of the culture medium has a profound influence on cell maturation (206). Mature neurons appear preferentially in a serum-free environment, suggesting that the absence of certain mitogens in the serum favors neuroblastoma cell differentiation (206,207). Table 3 provides a summary of agents capable of enhancing the neuronal maturation of neuroblastoma cells in vitro. The most widely studied class of agents are those that increase cytoplasmic cAMP levels in neuroblastoma cells. The mechanism of action of nonpolar solvents is not clear, but presumably they act at the level of the cell membrane (201). As was noted for EC cells, drugs that inhibit DNA synthesis also enhance neuroblastoma cell maturation (table 3). Agents in all of these three categories appear to be capable of inducing neurite formation in at least one human neuroblastoma cell line (table 3A). Although most of the studies listed in table 3A report that cellular proliferation was decreased in conjunction with the morphological changes observed, very few of these investigations have ascertained whether tumorigenicity was simultaneously reduced by drug treatment (208). Exposure of C1300 cells in vitro to a combination of prostaglandin $E_1$ and the phosphodiesterase inhibitor RO20-1324, however, has been shown to produce a marked reduction in "tumor-takes" when treated cells were injected into mice, and injection of these two drugs around established neuroblastomas in mice led to tumor regression (89).

Neuroblastoma-bearing mice treated with a combination of dibutyryl cyclic AMP, papaverine, and theophylline showed a transient reduction in tumor growth (88). Helson et al (90,91) incorporated papaverine treatment in a combination chemotherapy protocol for childhood neuroblastoma, and observed responses in 14 of 15 treated children. However, the results were only compared to a historical control group treated with a combination of two cytotoxic drugs. Although these preliminary in vivo studies suggest that agents that increase cyclic AMP levels may possess antitumor effects, evidence is lacking that this effect can be ascribed to the induction of maturation. Thus, it is also conceivable that the treatment unmasks or induces cell surface antigens to which the host mounts an immune response (209).

Table 4 lists experimental information that is available on the control of maturation of cells derived from epithelial tumors. The same classes of agents known to induce the differentiation of teratocarcinoma cells, ie, polar solvents, retinoids, drugs that increase intracellular cAMP, and a number of cytotoxic agents, have been shown to alter the morphology and biochemical characteristics of both murine and human melanoma cells (93–96). These cells display dendrite formation, increased tyrosinase activity, and enhanced melanin production. These quantitative changes, however, do not necessarily correlate with the irreversible loss of proliferative potential and, indeed, careful studies by Bennett (210) on single cells suggest that an increase in melanin production may be a reversible phenomenon.

DeCosse et al (149) reported in 1973 that mouse mammary adenocarcinoma cells form tubules, with a concomitant decline in DNA synthesis, when exposed to embryonic mesenchymal tissue. Subsequently, "dome and ridge" formation (97) and the secretion of immunoreactive casein (98) were described as evidence of differentiation of rat breast carcinoma cells in vitro. Both parameters of maturation could be greatly enhanced by prostaglandin $E_1$ ($PGE_1$), DMSO, and retinoic acid (99). That true terminal differentiation was achieved was suggested by the fact that in vivo tumorigenicity of Rama 25 cells pretreated with DMSO or $PGE_1$ was significantly reduced (98).

A second type of adenocarcinoma that has received attention as a model for tumor cell differentiation is carcinoma of the colon. Dexter and Hager (100) and Hager et al (101) reported that exposure of two human colon carcinoma lines (HCT-15 and DLD-1) to *N,N*-dimethylformamide led to a decrease in clonogenicity in vitro as well as in tumorigenicity in nude mice. The expression of carcinoembryonic and colonic mucoprotein antigens on the cell surface was used as markers of maturation. Further-

more, growth of HCT-15 and DLD-1 tumors transplanted into nude mice was inhibited by the treatment of animals with dimethyl- or methyl-formamide (211). Kim et al (102) reported that DMSO and sodium butyrate inhibited the growth of two other human colonic adenocarcinoma lines (SW 480, SW 620); however, in this instance, tumorigenicity was not affected. Although alterations in cell morphology and cell surface ganglioside composition were taken to represent aspects of terminal differentiation, these parameters appeared to be relatively nonspecific. Recently, the HT-29 cell line derived from a human colon carcinoma was shown to be induced to terminal differentiation by removal of glucose from the medium (103), as well as by exposure to sodium butyrate (104). The criteria for maturation included dome formation, mucus secretion, and ultrastructural morphology (cuboidal simple epithelium with brush border typical of colonic mucosa). Clonal lines were established which retained the ability to mature to variable degrees (104). The HT-29 cell line may prove to be a suitable model to assay the activity of potential inducers of the differentiation of colon carcinoma.

Carcinomas derived from stratified squamous epithelia eg, naso- and oro-pharyngeal mucosa, esophagus, bronchus, cervix uteri) represent approximately 15% of all malignant solid tumors, excluding skin carcinomas. Stem cells in nonneoplastic epidermis can only differentiate along a single pathway, which results in a layer of fully keratinized cells. As maturing keratinocytes move up through successive cell layers in the epidermis, the spatial organization of the tissue reflects the time-dependent changes in cell maturation. Thus, the bottom basal layer contains stem cells, and the upper ones contain the maturing cells. Several aspects of keratinization have been studied extensively. Specific alterations in keratin polypeptides, the synthesis of involucrin and other precursors of the cornified cell envelope, and changes in cell surface glycoproteins represent major parameters of cell maturation (212). Normal keratinocytes can be cultured in vitro, and thus provide an excellent framework with which to compare the behavior of neoplastic squamous cells. Although no experimental in vivo model is currently available for investigations on the growth and maturation of squamous carcinomas, current studies on human pathology specimens are showing that the pattern of keratins and envelope precursor(s) are detectable in these tumors and are comparable to their nonneoplastic tissue of origin (213). Although differentiation to nontumorigenic keratinized cells occurs in these tumors to some extent in vivo (120), several lines of evidence suggest that this process is abnormally regulated compared to that occurring in normal stratified squamous epithelium. Thus, Frankfurt (122) has shown that the rate of terminal differentiation in tumors of the forestomach of the rat was decreased compared to nonneoplastic epithelium. Furthermore, Rheinwald and Beckett (105) showed that deprivation of anchorage, which rapidly leads to terminal differentiation of normal epidermal keratinocytes, resulted in a much slower rate of maturation and loss of clonogenicity of cell lines derived from squamous carcinomas of the head and neck region. Recently, it has become apparent that the loss of inducibility to terminal differentiation of epidermal keratinocytes is an early event in the process of chemical transformation (123). We have recently shown that cells derived from a human squamous carcinoma of the oral mucosa (SqCC/Y1) can be induced to keratinize in vitro by cultivation in a medium largely devoid of mitogens and retinoids (107). Under these conditions, not only do individual cells become keratinized and form cornified cell envelopes, but a complex multilayered cellular structure develops which has many of the morphological (ultrastructural) characteristics of normal stratified squamous epithelium. Importantly, the appearance of mature cells is accompanied by a decrease in clonogenicity. Moreover, the observations of Cline and Rice (106), using another cell line of similar origin, that retinoids inhibit squamous differentiation, whereas hydrocortisone appears to antagonize this effect, were confirmed by our laboratory with SqCC/Y1 cells (214).

In summary, cell lines derived from most types of malignant tumors of epithelial origin have been established which display some characteristics of terminal maturation in vitro under appropriate conditions. Phenotypic characteristics of mature cells which clearly distinguish them from tumor stem cells have been most extensively described for germ cell tumors and epidermoid carcinomas. Both models could well be utilized for preclinical testing of potential inducers of differentiation, although embryonal carcinoma cells are multipotent and thus appear to represent a much more complex model than squamous cell carcinoma. Moreover, epidermoid carcinomas are much more prevalent than germ cell tumors; thus, results obtained with these neoplastic cell lines may have greater relevance for application in the clinical situation. Neuroblastomas are the most prevalent malignant tumor in childhood, but rarely appear in adults; the applicability of drug studies with this tumor would appear, therefore, to be limited to pediatric oncology. Investigations conducted to date indicate that different drugs are required to induce differentiation in different tissues. An example of this phenomenon is that retinoids, which enhance maturation in the leukemias, teratocarcinomas, and neuroectodermal tumors, inhibit keratinization in epidermoid tumors. Thus, it will probably be necessary to establish in vitro and animal models for each of the major types of malignant tumors to effectively evaluate potential inducers of differentiation.

## CONCLUSION

This report reviews some of the evidence which demonstrates that the conversion of self-renewing malignant

stem cells into terminally differentiated cells is inhibited or abnormally regulated in both hematological malignancies and solid tumors. This abnormality in the control of the maturation of neoplastic cells contributes to the relative increase in the stem-cell pool, and thus to the growth of the tumor. However, examples can be found for most types of malignancy which indicate that neoplastic stem cells have not irreversibly lost the ability to undergo a program of terminal differentiation to mature cells resembling those of the tissue of origin. The abnormality which is reflected as an imbalance in the number of self-renewing cells relative to functionally mature cells can be circumvented by (*a*) employing appropriate culture conditions for cells in vitro (neuroblastoma, squamous carcinomas); (*b*) reconstructing a normal extracellular environment (teratocarcinoma); or (*c*) using physiological inducers such as prostaglandins and retinoids or pharmacological agents such as polar-planar solvents and cytotoxic agents. When used in combination, antineoplastic agents and conditioned medium, which most likely supplies humoral factors, induce differentiation of leukemic cells in vitro in an additive or synergistic manner (30,189). Thus, a combination of circulating humoral factors with agents capable of initiating differentiation may produce significant therapeutic response. A variety of in vitro and in vivo models will be required to provide adequate evaluation of potential differentiating agents, because none of the tumor systems in which differentiation can be induced fulfill all of the criteria deemed important for an optimal model. Often, as is the case for melanoma and adenocarcinoma, the phenotypic characteristics do not allow a sharp distinction to be made between stem cells with infinite self-renewal potential and committed cells destined to terminate proliferation. In other cases, no in vivo transplantable form of the tumor is available in which the effect of drugs can be assessed in animals. In contrast, in vivo models of leukemia exist both in the mouse (153,187) and rat (190) that would allow the screening of agents that function to control tumor growth through differentiation. This type of model permits assessment of the role of the microenvironment and auxiliary cells in relation to the leukemic process. It is also important to evaluate the response of both leukemic and normal cells that are located within organs that act as drug depots (4). A more fundamental objection to the use of a single in vitro screening system for chemicals that induce maturation is that a given agent can affect the process of differentiation differently in various types of tumors. This is most clearly demonstrable for the retinoids, which induce maturation of tumor cells derived from mesenchymal (19) or neuroectodermal origin (93), variably affect germ-line tumors (54,204), and inhibit maturation of squamous cell carcinomas (106,107). The currently available spectrum of experimental tumors of both animal and human origin, derived from solid cancers as well as from hematological malignancies, should enable a reasonable preclinical evaluation of differentiating agents with potential clinical usefulness.

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# Biochemical Modulation: Application of Laboratory Models to the Clinic

**Brian Leyland-Jones* and Peter J. O'Dwyer[1,2]**

The active anticancer drugs in clinical use today are distinguished by their low therapeutic index and poor selectivity for tumor over normal tissue. Despite this shortcoming, chemotherapeutic agents, especially when used in combination regimens, are having an increasing impact on the palliation and cure of selected tumors. Much recent research has been aimed toward increasing the therapeutic index of these drugs. Strategies based on the concept of biological modulation are being employed increasingly for this purpose.

Biochemical modulation consists of the use of one or more agents, which may themselves be devoid of cytotoxic activity, to enhance the antitumor effect and/or the selectivity of an active antitumor drug. The interaction of the modulating agent and the active drug may be at a variety of points between the plasma and the intracellular target. Such interactions are diverse, both mechanistically and in potential clinical application, for example:

(*a*) modulation of antimetabolites by other antimetabolites—5-FU (by methotrexate, PALA, or leucovorin) and cytarabine (araC) [by PALA, thymidine (TdR), or acivicin];

(*b*) modulation of DNA-reactive agents—alkylating agents by chemoprotectors and anthracyclines by agents which act on cellular calcium metabolism (verapamil and trifluoperazine);

(*c*) modulation of DNA repair by depletion of critical intermediates—6-aminonicotinamide, which inhibits poly-ADP ribosylation;

(*d*) modulation of compounds which act through reactive oxygen intermediates by agents which deplete cellular glutathione—buthionine sulfoximine;

(*e*) protection of normal tissues from drug toxicity—cisplatin and thiosulfate or diethyldithiocarbamate, 5-FU, and uridine; and

(*f*) modulation of radiation toxicity by agents which sensitize tumor tissues—nitroimidazoles.

These leads have not, however, been successfully applied to clinical trials in a rational and systematic manner. Using examples drawn from the modulation of antimetabolites, this paper will trace the basic assumptions and preclinical concepts of biochemical modulation and suggest how these might be most rationally applied to the clinic.

A number of assumptions underlie the development of regimens based on biochemical modulation. One assumption is the issue of tumor cell heterogeneity. Strong evidence exists to support the existence of heterogeneous cell composition in solid tumors. The existence of a continuum of phenotypic manifestations of gene expression (G6PD, differentiation antigens), of ploidy and of malignant potential in the same tumor, stresses the dissimilarity of cells within a given tumor. The disparity in therapeutic response among tumors of a given type to an antitumor agent considered active against that type is attributed in large part to widely variable subpopulations of tumor cells with differing drug sensitivities within a single neoplasm (1-14). Combination chemotherapy was initiated primarily because of heterogeneity and evidence of differential drug sensitivities (15,16).

Few single agents yield response rates > 50% in any given tumor. The heterogeneity above can explain the resistance of most solid tumors to single agents which are "active" in the treatment of that tumor. Resistant clones may also arise as mutants during chemotherapy. Evidence from a variety of sources implicates a genetic basis for most drug-resistant phenotypes (17). Furthermore, the concept of treatment failure being related to the emergence of drug-resistant cancer cells during chemotherapy has been well-documented in the literature (18-23).

Combination chemotherapy has failed to overcome the clinical problems of latent and acquired resistance for many tumor types. The failure may be attributed in part to the presence of highly resistant clones; however, this resistance is relative and cytotoxicity is usually observed when the antitumor agent is present in sufficiently high concentration. Whereas host toxicity normally prevents administration of such concentrations, selective delivery to the tumor of a maximal *effective* dose of active agents represents a method for overcoming the relative resist-

[1]Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

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* *Reprint requests to:* Brian Leyland-Jones, MD, Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Landow Bldg, Rm 4C09, National Institutes of Health, Bethesda, MD 20892.

ance which is a consequence of heterogeneity. Enhancement of the receptor binding and selectivity of these doses is the goal of biochemical modulation.

### The Effector Agent

It should be emphasized that the principal aim of biochemical modulation (at present) is the modulation of the efficacy of a single agent, the latter being called the *effector agent*. Drugs such as PALA, verapamil, misonidazole, etc, have little antitumor effect in their own right, and may be called *modulating agents*. Other modulating agents (such as methotrexate and acivicin) may have antitumor activity; however, their use as modulating agents is independent of this and relies on the careful selection of dose and schedule to manipulate the cytotoxicity of an effector drug. Much of the original work in biochemical modulation has been directed at using these modulating agents to drive effector agents (eg, 5-FU or araC) into their effector sites with greater efficacy. This illustrates an important difference between combination chemotherapy and biochemical modulation. The former adds together drugs with established single-agent activity in a given tumor. Doses are modified only as dictated by the additive toxicity of the individual drugs, and schedules are empirically derived. Biochemical modulation seeks to increase the tumor-specific cytotoxicity of a known active drug by manipulation of the determinants of its cytotoxicity. This is achieved by the use of agents, which in doses and schedules chosen on the basis of biochemically determined findings will increase the therapeutic index of the drug.

The pyrimidine 5-FU was one of the first rationally synthesized anticancer compounds and is clinically active against a variety of tumors. Two different mechanisms of action have been postulated. First, DNA synthesis may be inhibited by the conversion of 5-FU to the metabolite 5-FdUMP, the latter binding irreversibly with the enzyme thymidylate synthetase and thereby inhibiting the synthesis of dTMP (24). The second mechanism involves the RNA pathway: incorporation of the 5-FU metabolite 5-FUTP results in disruption of RNA synthesis and function (24,25). Although both of these mechanisms may be important, strong experimental correlations have been made between the antitumor effects of 5-FU and 5-FUTP incorporation in RNA (26-28).

At least two methods may be employed to enhance the incorporation of 5-FUTP into RNA. One approach is the reduction of uracil nucleotide pools, which will reduce competition for incorporation of 5-FUTP. PALA is a transition-state analog inhibitor of the enzyme aspartate transcarbamylase which catalyzes an early step in the de novo synthesis of uridine (30). PALA has some cytotoxic activity itself in vivo, especially against B16 melanoma and Lewis lung carcinoma, but is inactive in the $CD8F_1$ mammary tumor. In this last model, however, the combination of a low nontherapeutic, but biochemically active, dose of PALA and 5-FU in vivo yields antitumor activity greater than the maximally tolerated dose of 5-FU alone; the enhanced activity correlates with increased incorporation of 5-FUTP into RNA (31-33).

A second approach is to use modulating agents to increase the intracellular levels of 6-methylmercaptopurine riboside (PRPP), which is a critical cofactor for the synthesis of 5-FUMP, the precursor of 5-FUTP. Increasing the anabolism of 5-FU along this ribonucleotide pathway increases the availability of the active metabolite for incorporation into RNA. Methotrexate increases PRPP levels, enhances incorporation of 5-FU into RNA, and potentiates the cytotoxicity of 5-FU in L1210 cells (26). MMPR, an inhibitor of the first enzyme in purine synthesis, amidophosphoribosyltransferase (34,35), also increases PRPP levels, and similarly potentiates antitumor activity of 5-FU (25,32,33).

Thus, a number of drugs, exclusive of their antitumor activity in the models tested, may potentiate the cytotoxicity of 5-FU through their effects on the metabolic pathways which activate 5-FU. The improved survival and cure rates in these in vivo models demonstrate further that the selectivity of 5-FU for tumor tissue has been enhanced by these influences.

AraC is one of the most effective agents in the treatment of acute myeloblastic leukemia; however, the basis for its selectivity against leukemic cells remains unclear. The drug is an analog of deoxycytidine, and in phosphorylated form competes with dCTP for sites on DNA polymerase and for incorporation into DNA. Both of these actions have been postulated to be responsible for the drug's cytotoxicity (36,37). Recent studies have favored the latter effect as being more important (38). In either case, drug activity is based on competition with dCTP, and successful competition is therefore dependent on the cellular concentrations of both dCTP and araCTP. Rustum and Priesler (39) demonstrated a correlation between patient response and leukemic cell araCTP retention in vitro. Iacoboni et al (40) have extended these findings, relating araCTP levels in circulating blasts to outcome of therapy with high-dose araC. Cellular dCTP levels are important not only because of competition with araCTP, but also because increased levels of dCTP inhibit deoxycytidine kinase, the enzyme responsible for the initial phosphorylation of araC (41). When cellular dCTP levels are diminished by treatment of cells in culture, deoxycytidine kinase activity increases, leading to increased araCTP production and enhanced cytotoxicity (42). Kufe et al (38) have demonstrated that incorporation of araC into DNA is the single most powerful predictor of cell lethality. The highly significant relationship between extent of (araC) DNA formation and inhibition of DNA synthesis and the relative chain-terminating effect of this drug strongly suggest that incorporation into the DNA strand is responsible for inducing lethal cellular events (42). Hence, the efficacy of araC should be increased by biochemically modulating cellular araC

metabolism in such a way that the cellular accumulation and retention of tumor araCTP are selectively enhanced.

Pretreating cells with agents that decrease cellular dCTP levels results in greater cytotoxicity of araC. Such agents include PALA and TdR, both of which act on different steps in the biochemical synthetic pathway of dCTP. PALA achieves this by inhibition of the de novo synthetic pathway of pyrimidine nucleosides (43). TdR has many cellular effects, including the ability of its triphosphate, dTTP, to inhibit ribonucleotide reductase and thereby decrease levels of dCTP (44). As will be described later, these agents have been used alone in the treatment of cancer patients and have been found to be nontoxic, while eliciting the predicted biochemical response. It is therefore rational to use these agents, based on their relative lack of toxicity and ability to enhance cellular araCTP levels by differing mechanisms, to improve the activity of araC in the clinic (45).

## Modulating Agents

We will now turn our attention to drugs that modulate the effector agents. As previously stated, these drugs need not necessarily have antitumor activity of their own. We will concentrate on the use of two agents, PALA and TdR, since these agents have already been shown to have insignificant clinical antitumor activity of their own, yet show significant promise as biochemical modulators in preclinical models.

## PALA

As stated above, PALA inhibits the second enzyme in the de novo pyrimidine biosynthetic pathway (30,43,46). The cytotoxic and cytostatic effects of PALA on the growth of cells in culture have been shown to be reversed by uridine; moreover, the toxicity and antitumor activity of the drug can be reversed by uridine or *N*-carbamyl-DL-aspartate in mice, showing, in both cases, that the effects of PALA are due specifically to the blockade of de novo pyrimidine nucleotide biosynthesis (43,46–48).

PALA inhibition of pyrimidine synthesis results in a lowering of intracellular UTP pools (49–53). Thus, when PALA is administered prior to 5-FU, the lowered UTP pools reduce competition for FUTP utilization by RNA polymerase, resulting in increased incorporation of 5-FU into tumor RNA (54–56). Pretreatment with PALA has demonstrated enhanced antitumor activity in several models (25,27,29,32,57,58).

PALA is cytotoxic both in vitro and in vivo (51,59–61). One important determinant of the cytotoxicity of PALA is the level of ACTase activity: naturally occurring resistance to the drug has been associated with high ACTase activity (59,61,62). Other factors resulting in diminished sensitivity to PALA include high activity of the target tumor cell's pyrimidine salvage pathway or high levels of carbamyl phosphate at the active site of ACTase (59,62,63).

In human tumors, tumor ACTase activities were found to be substantially lower than those observed in experimental tumors (64,65), suggesting increased sensitivity to PALA. Furthermore, inhibition of ACTase by a single dose of PALA lasts for several days in both mice (32,52,53,66) and man (64,67,68). These two facts suggest that a single low dose of the modulating agent PALA could be effective in increasing the antitumor activity of a maximally tolerated dose of the effector agent 5-FU.

### *Rationale for Combining PALA and 5-FU*

As stated, PALA lowers intracellular levels of UTP, leading to reduced competition for incorporation of 5-FUTP into nucleic acids. PALA pretreatment has been demonstrated to increase 5-FU incorporation into tumor cell RNA in both murine and human tumor cell lines (69,70). The synergistic antitumor activity of PALA and 5-FU has been demonstrated in several murine solid tumors (29,55,69–71).

The fact that initial clinical trials of PALA and 5-FU in man were disappointing (table 1) demonstrates the importance of the concept of modulating and effector agents. In all of these studies, a high dose of the modulating agent PALA was used (1–2 g/m$^2$). The clinical toxic effects of the modulating agent seen at this dose prevented the administration of full doses of the effector agent 5-FU. Since PALA has no observable clinical antitumor activity of its own, and the dose-response curve for 5-FU is steep, it is not surprising that a high PALA/low 5-FU combination demonstrated poor clinical activity.

Casper et al (77) have demonstrated that a dose of 250 mg/m$^2$ of PALA is sufficient to inhibit total-body pyrimidine synthesis. In a phase I trial, incorporating this dose of PALA and escalating doses of 5-FU, 750 mg/m$^2$ of 5-FU was attainable with tolerable toxicity. This dosage ratio of high effector and low modulating agent ratio is the most desirable for further clinical development. Furthermore, it is hoped that such a combination would achieve selectivity based on the lower concentration of PALA's target enzyme, aspartate transcarbamylase, in tumor than in normal tissues (78).

## Thymidine

### *PALA/TdR/5-FU*

The rationale for the combination of PALA, 5-FU, and TdR is that both TdR and PALA enhance the formation of FUTP and its incorporation into RNA. The biochemical interaction between 5-FU and PALA was discussed earlier. TdR may increase 5-FU incorporation into RNA via two mechanisms: (*a*) an excess of TdR produces feedback inhibition of several enzymes in the de novo nucleotide pathways (one site of inhibition may be at the level of the conversion of 5-FU to FdUMP, resulting in the

TABLE 1.—Clinical trials with PALA and 5-FU

| Institution | Disease | PALA Dose ($g/m^2$) | PALA Schedule | 5-FU Dose (mg) | 5-FU Schedule | No. of patients | No. of complete + partial responses | Ref No. |
|---|---|---|---|---|---|---|---|---|
| M. D. Anderson | Colon cancer | 2 | Weekly | 480 | Every week | 24 | 4 | 72 |
| M. D. Anderson | Colon cancer | 0.8 | × 5 days | 400 | × 5 days | 27 | 3 | 72 |
| Dana-Farber Cancer Institute | Breast and colon cancer | 0.85 | × 5 days | 300 | × 5 days | 43 | 6 | 73 |
| City of Hope | Breast and colon cancer | 0.94 | × 5 days | 325 | × 5 days | 18 | 6 | 74 |
| National Cancer Institute | Melanoma | 1.5 | × 5 days | 150 | × 5 days | 18 | 2 | 75 |
| Eastern Cooperative Oncology Group | Colon cancer | 1.0 | Single dose | 400 | Days 1 and 8 | 17 | 1 | 76 |

channeling of 5-FU into FUTP and hence RNA); and (*b*) TdR undergoes rapid conversion to TdR, which inhibits 5-FU catabolism and markedly prolongs its half-life. In an in vivo study, Martin et al (79,80) observed that prior treatment with TdR increased the antitumor activity of 5-FU in $CD8F_1$ murine mammary carcinoma and in Colon tumor 26. In vitro, an enhanced cytotoxic effect of 5-FU was observed in AKR leukemia colony-forming units if TdR was given 1 hour before 5-FU (81).

Again, if clinical trials are to mimic the preclinical concepts, they should maximize the dose of the effector agent (5-FU) and use only modulating doses of the other two agents. Table 2 shows the PALA/TdR/5-FU trials conducted to date. It will be seen from these trials, which were largely phase I dose-finding studies, that the dose of PALA was high, the dose of TdR empirical, and consequently, the dose of 5-FU far below that which would ordinarily be tolerated as a single agent. This illustrates the need for further careful studies, before it can be appropriately evaluated in phase II-III trials.

### *PALA/TdR/AraC*

The inhibition of ribonucleotide reductase by dTTP following administration of TdR also diminishes intracellular pools of dCTP (86). Competition for incorporation of araCTP into DNA is thereby reduced. Further, low levels of dCTP release the feedback inhibition of deoxycytidine kinase and permit greater activation of araC to its triphosphate (87,88).

Three clinical trials of combined TdR and araC have been conducted (table 3). In the study by Van Echo et al (89,90), TdR administration preceded that of araC by 2 hours; thereafter, the drugs were administered concurrently to marrow aplasia. TdR doses of 8-40 $g/m^2$/day were used. In addition to myelotoxicity, mucositis, der-

TABLE 2.—Studies of the modulation of 5-FU by PALA and TdR

| Investigator (Ref No.) | Study design | Drug | Dose | Schedule | Toxic effect | Comments |
|---|---|---|---|---|---|---|
| Casper et al (82) | Phase I | PALA | 0.25-2 $g/m^2$ | Day 1 | CNS; gastrointestinal myelosuppression (mild) | CNS toxicity prevented delivery of adequate dose of effector agent |
| | | TdR | 45 g | 90-min infusion on Day 2 | | |
| | | 5-FU | 100-150 $mg/m^2$ | Rapid infusion 30 mins after beginning TdR | | |
| Chiuten et al (83) | Phase I | PALA | 1 $g/m^2$ | Day 1 | Myelosuppression; gastrointestinal; CNS | |
| | | TdR | 30 g | Rapid infusion on Day 2 | | |
| | | 5-FU | 150-250 $mg/m^2$ | Simultaneous rapid infusion on Day 2 | | |
| Basseches et al (84) | Phase I | PALA | 4 $g/m^2$ | Day 1 | Myelosuppression; gastrointestinal | |
| | | TdR | 15 g | 30-min infusion on Day 2 | | |
| | | 5-FU | 100-200 $mg/m^2$ | 30 mins after TdR on Day 2 | | |
| O'Connell et al (85) | Phase II | PALA | 4 $g/m^2$ | Day 1 | Myelosuppression; CNS | 10 of 37 patients responded; 6 of 13 responders among patients with anaplastic histology |
| | | TdR | 15 g | 30-min infusion on Day 2 | | |
| | | 5-FU | 200 $mg/m^2$ | 30 mins after TdR on Day 2 | | |

TABLE 3.—Phase II clinical trials of araC modulation by TdR in leukemia

| Investigator (Ref No.) | Drug | Dose | Schedule | No. of evaluable patients | No. of complete responses | Toxic effect* |
|---|---|---|---|---|---|---|
| Van Echo et al (89,90) | TdR | 8-40 g/m²/day | Continuous infusion; initiate araC at 2 hrs | 41, ANLL<br>4, CML† | 12<br>1 | Gastrointestinal; skin rash; mucositis; hepatic |
| | AraC | 250 mg/m²/day | Continuous infusion; continue until aplasia (7-12 days) every 4-8 wks | | | |
| Blumenreich et al (91) | TdR<br>AraC | 30-75 g/m²/day<br>200 mg/m²/day | Days 1, 3, and 5<br>Days 2, 4, and 6 | 6 | | Mild CNS, gastrointestinal; hepatic; mild |
| Zittoun et al (92) | TdR<br>AraC | 30 g/m²/day/× 1<br>200 mg/m²/day/× 2 | Continuous infusion<br>Days 4-9 | 6, ANLL<br>5, CML | 2 | Gastrointestinal; mild CNS |

*Dose-limiting toxicity.
†CML = chronic myelogenous leukemia.

matitis, and hepatitis were prominent. However, neither time to aplasia nor the incidence of these toxic effects appeared to be related to the dose of TdR, and all doses were well-tolerated. In the patients with acute nonlymphocytic leukemia (ANLL), almost all of whom had relapsed on or had failed induction with araC-containing regimens, 12 of 41 achieved complete remission. The incidence of complete remission was not related to the dose of TdR. Similar (but less severe) toxicity was observed in the study of Blumenreich et al (91), in which TdR administration preceded that of araC by 5-8 days. Two of six patients with ANLL achieved complete remission. Flash-labeling of bone marrow cells with araC and deoxycytidine did not demonstrate conclusively that enhancement of araC incorporation had occurred. Zittoun et al (92) used a less intensive regimen and observed complete remissions in two of five patients with chronic myelocytic leukemia and in two of six with ANLL.

Preclinical studies indicate synergy between TdR and araC in vitro and in vivo (86,93). However, in this instance, the importance of appropriate scheduling of the agent is illustrated in the preclinical studies. Simultaneous infusion of TdR and araC in vitro produced maximal increase in the biologic activity of araC in L1210 leukemia (88). In Fisher rats, araC activity was potentiated when araC was administered simultaneously with TdR, following a 24-hour infusion with TdR alone. No potentiation was observed if the animals were not preinfused with TdR (93).

These studies suggest that TdR/araC combinations may have clinical utility in patients who have failed araC-containing regimens. However, it is still not known how TdR and PALA may be used optimally to selectively modulate araC in the clinic. High-dose araC has also shown promise in such patients. Clarification of the biochemical interactions between modulators and araC and comparison with data generated in high-dose araC studies may indicate how the therapeutic index of araC may be enhanced by such modulation.

In summary, therefore, these examples of modulatory combinations serve to emphasize the importance of dose and schedule, both of the effector and of the modulator, in achieving optimal synergy. Because of the low therapeutic index of cytotoxic drugs, failure to observe the experimental conditions of the preclinical studies carefully may abolish the synergy sought from such combinations. Hence, the development of regimens involving biochemical modulation should follow, where possible, a series of rational steps to assure the replication in the clinic of promising results obtained in preclinical models.

## Logical Progression From In Vitro to In Vivo to Clinic

The preceding section serves to further highlight two of the important issues in successful biochemical modulation: dose-response and sequence of drug administration. We have mentioned the dose-response relationship of PALA to UTP pools in $CD8F_1$ tumor-bearing mice. Good reduction of pools occurs at a dose of 100 mg/kg, which is nontherapeutic and nontoxic in those animals. Using a technique developed by Moyer and Handschumacher (94) to determine the effective dose of PALA which blocks whole-body de novo pyrimidine synthesis, Casper et al (77) showed that 250 mg/m² was maximally effective. In translating this to man, one would therefore anticipate that higher doses of the modulating agent would not increase the desired biochemical effect, and moreover would reduce tolerable dose of the effector agent because of overlapping toxicity. Therefore, the maximally effective rather than the maximally tolerated PALA dose should be applied to modulation studies.

The importance of appropriate sequencing is best exemplified by the biochemical modulation of 5-FU by methotrexate. Synergy in several tumors occurs when methotrexate precedes 5-FU, but not when the order is reversed. Table 4 shows in vivo studies of methotrexate scheduling in murine tumors by Mulder et al (95). Responses as shown by growth delay in the osteosarcoma model and by percent increase in lifespan in the L1210

TABLE 4.—Effect of 5-FU/methotrexate drug scheduling in osteosarcoma- and in L1210 leukemia-bearing mice*

| Order of drug administration | Interval between drugs (hrs) | Osteosarcoma growth delay (days) | L1210 leukemia (% increase in life span) |
|---|---|---|---|
| 5-FU + methotrexate | 24 | 3.2 | 39 |
| 5-FU + methotrexate | 16 | 2.0 | 38 |
| 5-FU + methotrexate | 3 | 2.0 | 40 |
| 5-FU + methotrexate | 1 | 2.7 | 38 |
| 5-FU + methotrexate | 0 | 3.3 | 42 |
| methotrexate + 5-FU | 1 | 3.4 | 52 |
| methotrexate + 5-FU | 3 | 3.5 | 42 |
| methotrexate + 5-FU | 16 | 4.2 | 55 |
| methotrexate + 5-FU | 24 | 5.5 | 55 |

* *From* ref 95.

leukemia model increased with the interval between methotrexate and 5-FU to reach a maximum at 24 hours. In a human colon tumor in vitro, Benz and Cadman (96) have confirmed that at least 18 hours must elapse between methotrexate and 5-FU exposure for maximal enhancement of 5-FU incorporation into RNA. Therefore, though biochemical confirmation of this relationship at an in vivo level is lacking, the data strongly support an interval of at least 24 hours between methotrexate and 5-FU administration in clinical studies. Table 5 represents a compilation of clinical trials in advanced colorectal cancer of sequenced methotrexate and 5-FU performed to date. Unfortunately, despite the evidence that a long time interval should maximize synergy, most of these studies have been conducted at suboptimal scheduling intervals. A randomized trial of two regimens of methotrexate/5-FU sought to determine the importance of scheduling. One hundred eight patients (including 70 with squamous cell carcinoma of the head and neck) were treated with methotrexate followed *1 hour* later by 5-FU or the same drugs administered in reverse order (97). There was no difference in response rates or survival in the two arms; indeed, the 5-FU/methotrexate arm appeared to be superior for one subgroup. However, because of the short time intervals chosen, it must be concluded that a definitive study of the role of the scheduling interval in the combination of methotrexate prior to 5-FU has not yet been performed.

TABLE 5.—Results of trials of sequential methotrexate/5-FU in colorectal cancer

| Investigator (Ref No.) | Methotrexate ($mg/m^2$) | Interval (hrs) | 5-FU ($mg/m^2$) | No. of responders/ No. of patients treated |
|---|---|---|---|---|
| Rangineni et al (98) | 60 | 0 | 1500 | 1/30 (3%) |
| Biran et al (99)* | 30 | 0 | 600 | 0/31 (0%) |
| Mahajan et al (100) | 800 | 0 | 800 | 2/13 (15%) |
| Tisman and Wu (101) | 1500 | 1 | 1500 | 2/7 (29%) |
| Burnet et al (102) | 250 | 1 | 600 | 1/18 (6%) |
| Cantrell et al (103) | 250 | 1 | 600 | 1/16 (6%) |
| Blumenreich et al (104) | 200 | 1 | 600 | 0/7 (0%) |
| Panasci and Margolise (105) | 200 | 1 | 1000 | 2/9 (22%) |
| Panasci et al (105) | 200 | 1 | 600 | 0/5 (0%) |
| Coates et al (106) | 250 | 1 | 600 | 3/5 (60%) |
| Hansen et al (107) | 100 | 1 | 600 | 5/20 (25%) |
| Friedman et al (108) | 250 | 2 | 1000 | 2/12 (17%) |
| Mahajan et al (100) | 800 | 3 | 800 | 2/17 (12%) |
| Mehrotra et al (109) | 100 | 4 | 600 | 8/10 (80%) |
| Weinerman et al (110) | 20 (mg/kg) | 4 | 600 | 10/29 (34%) |
| Solan et al (111) | 40 | 4 | 600 | 3/8 (38%) |
| Solan et al (111) | 200 | 4 | 600 | 2/6 (33%) |
| Moayeri et al (112) | 200 | 7 | 600 | 6/26 (23%) |
| Hermann et al (113) | 200-300 | 7 | 900 | 5/8 (63%) |
| Drapkin et al (114) | 200-600 | 7 | 300-600 | 5/19 (26%) |
| Ignoffo et al (115)* | 60 | 12 | 500 | 19/49 (39%) |
| Kemeny et al (116) | 40 | 24 | 600 | 14/43 (33%) |

* Mitomycin given in addition to methotrexate/5-FU.

TABLE 6.—Biochemical modulation development plan for modulatory combinations

1. Establish the mechanism of the modulation at a molecular level in an in vitro setting.
2. Measure the efficacy of the combination using conventional antitumor screens in vitro.
3. Confirm the enhanced efficacy, and refine it at an in vivo preclinical level.
4. Take the combination into phase I clinical testing, and at that level:
   a. Establish that the projected modulation is indeed occurring in the target tissue.
   b. Examine the pharmacokinetics and pharmacodynamics of the combination for advancement to phase II trials.
   c. Examine the alterations in the selectivity by the modulation at a biochemical and clinical level.

TABLE 7.—Biochemical modulation clinical trials

A. Phase I trial to:
   Confirm modulation
   Describe pharmacokinetics and pharmacodynamics
   Demonstrate selectivity
   Establish maximum tolerated dose of drug being modulated
B. Phase II trials to:
   Establish if response rate is not acceptable (25–30 patients)
   Determine appropriate tumor types for further study
C. Phase III trials to:
   Determine if modulation of the active drug confers an advantage:
      X - Y vs Y
      A - X - Y vs A - Y
   Need narrow confidence limits to detect a 15% improvement in efficacy (150–300 patients)
   Must be conducted in the appropriate patient population and tumor type

The examples of PALA/5-FU and methotrexate/5-FU may be extended to other combinations which have been examined in the clinic. Their common feature is that the majority of clinical trials have been conducted on the basis of synergy data in vitro without refining the methodology based on the dose and scheduling information obtained in vivo. In animal models, we have seen how the evaluation of PALA/5-FU and methotrexate/5-FU has indicated the manner in which the clinical trials should be conducted for optimal demonstration of synergistic effect. Failure to take these in vivo models (and in the case of PALA, biochemical information from initial clinical trials) into consideration leads to the inappropriate application of in vitro data to the clinic and the failure to demonstrate a therapeutic advantage.

These examples are taken to emphasize the importance of the logical progression of modulatory combinations through the in vitro and preclinical in vivo settings before clinical studies are undertaken. What is also desirable, although it was lacking in the initial clinical studies of both of these regimens, is appropriate biochemical measurements to ensure that modulation is indeed taking place. Although the difficulty in measuring intermediates such as PRPP levels in vivo is not to be underestimated, it is certainly feasible to do so in animal tumors. Furthermore, incorporation of radiolabeled drug can be studied more expeditiously in animals. This is not to diminish the importance of biochemical studies to accompany modulation studies in man, but rather to emphasize that the animal studies should be used to confirm and characterize the modulatory mechanism to allow the most appropriate and efficient studies to be performed on patients in clinical trials.

We would like to close by offering a schematic outline for the development of modulatory combinations (table 6). Clearly, both laboratory and clinical methods continue to evolve in this area, and such a schema requires tailoring to the combination being investigated.

The progression here is from in vitro to human studies. Where possible, in vitro studies should describe and characterize the mechanism of the modulation and its effect on cytotoxicity in tumor screens. Based on these studies, appropriate studies should be undertaken in *animal* models, first, to confirm that the appropriate modulation is occurring, and second, to define the parameters of the regimen which maximizes its selectivity. If the expected therapeutic advantage is still seen, then the regimen may be taken into the clinic. The initial human trial is the most difficult. Marked species differences in activities of metabolic pathways do not allow direct extrapolation of the animal model data to the clinic, although they may provide important guidelines. Hence, the initial human studies must (*a*) confirm that the modulation is occurring, (*b*) refine dose and scheduling for the human setting, and (*c*) determine the likely therapeutic advantage by examining both tumor and normal tissue. Clearly, for many agents not all steps will be feasible. However, phase III trials of such regimens require large numbers of patients; it therefore becomes crucial that the optimal doses and schedules be determined from the beginning.

This schema may be further extended into the clinical area. Table 7 reiterates what has already been discussed concerning phase I trials, except to add that a well-defined maximally tolerated dose is clearly important. Phase II trials are mentioned to minimize their importance. When one is attempting to modulate 5-FU or araC, one already has a good idea of expected response rates. The results in a small number of patients in an uncontrolled trial will not tell us if the modulation is useful. Its purpose is mainly to reassure that the regimen is tolerable for large-scale phase III testing. This step could indeed be omitted altogether by proceeding from phase I to phase III, with appropriate early stopping rules. The comparative study, however, must (*a*) be carefully designed, (*b*) be based strictly on the preclinical and early clinical data to which we have alluded, and (*c*) have endpoints which are sensitive enough to detect an advantage which is statistically demonstrable.

Appropriate designs for such trials will depend on the tumor type. For example, in head and neck cancer, for

which combination chemotherapy has not shown an advantage, PALA/5-FU versus 5-FU alone would be an appropriate study. In acute leukemia, on the other hand, it would be appropriate to add an anthracycline to each arm of a study modulating araC. The important feature of such trials, however, is that the statistical design be suitable for the demonstration of *likely* differences between the arms. For example, in a single agent versus a modulated single-agent trial, it is unreasonable to expect the modulation to increase response rates from 20% to 50%. In this setting, a real increase of just 15 percentage points would certainly be of interest. However, its demonstration may require 150 patients/arm.

It may be seen that the development of a combination with a modulatory rationale in the clinic requires the commitment of considerable resources. Ethical and practical reasons make it incumbent upon us to ensure that such combinations are given the best chance to work in man. This can only be assured if the preclinical and clinical development follows scientific criteria as rigorous as those applied at the bench.

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# ICIC NEWS

*The International Cancer Information Center (ICIC) is the focal point for dissemination of technical cancer information and has the goal of making physicians and scientists aware of the most recent and significant developments in the prevention and treatment of cancer and cancer etiology and biology. To this end, the ICIC develops and applies state-of-the-art computer and telecommunications technology to collect the results of cancer research and to disseminate this information for the benefit of physicians, cancer researchers, and other health professionals throughout the world. This information is provided through computer files (databases) and publications which are timely and easy to use.*

## CANCERLINE, A Comprehensive Cancer Information System, Available Through Your Biomedical Library

The CANCERLINE System consists of three separate databases. CANCERLIT contains approximately 500,000 citations and abstracts of published, international literature dealing with all aspects of cancer; it is updated monthly with approximately 5000 abstracts. CANCERPROJ contains about 5000 descriptions of current cancer research projects around the world. This file was reactivated in April 1985 and is expected to grow steadily with quarterly updating toward an eventual size of approximately 10,000 records. CLINPROT contains summaries of approximately 5000 clinical trials of new anticancer agents or treatment modalities. CLINPROT is updated monthly.

### Database Content

CANCERLIT

Approximately 80% of the literature in CANCERLIT is selected from an international collection of 3000 biomedical and scientific journals. Nonserial literature (including books, reports, and meeting abstracts) contributes the remaining 20%. Informative abstracts averaging 200 words are included for most selected cancer-related documents. The database includes cancer literature from 1963 forward. Literature available for the period 1963 through 1976 is limited to references that had been selected for *Carcinogenesis Abstracts* and *Cancer Therapy Abstracts*. In 1977, the scope of the database was expanded to include all cancer-related literature, except most single case histories. In 1980, new records began including MeSH terms for uniform retrieval, and since January 1985, new records have been indexed with chemical names and CAS Registry/EC Numbers.

CANCERPROJ

CANCERPROJ contains descriptions of federally and privately supported grants and contracts. Twenty percent of the project descriptions are provided by scientists from outside the United States. The project summaries are usually divided into a statement of the research objective, a description of the experimental approach, and a statement of any progress made to date.

CLINPROT

Most of the protocols in CLINPROT are provided by the Division of Cancer Treatment of the US National Cancer Institute, while the remaining protocols are provided by major US cancer centers or sources outside the United States. Protocol summaries include the objective and an outline of the study, patient entry criteria, dosage schedules, dosage forms,

and special study parameters. The name and telephone number of the study group chairman are provided. CLINPROT is designed primarily as a reference tool for clinical oncologists.

### Access to Cancerline

For a Search: CANCERLINE is accessed by computer terminals connected to the central computer facility located at the National Library of Medicine (NLM) in Bethesda, Maryland. These terminals are located at more than 2800 MEDLARS centers throughout the United States and in 14 foreign countries. Searches can be submitted at any of these locations. The requested information can be viewed at local terminals or printouts can be mailed from the computer center the following day. Many of these MEDLARS centers will search CANCERLINE on a "fee for services" basis for individuals who do not have their own computer terminal to access the databases.

For Subscription: If your organization would like to have the opportunity to search these files from its own computer terminal, you must apply for permission to the MEDLARS Management Section of the NLM at the following address:

National Library of Medicine
MEDLARS Management Section
8600 Rockville Pike
Bethesda, Maryland 20894
(301) 496-6193

Once accepted, your search analyst or librarian will receive formal training and access identification code. If you are a major US cancer treatment center, you may apply through the National Cancer Institute's International Cancer Research Data Bank Branch. Individuals may also apply for and receive access identification codes. Health professionals are eligible for a concentrated 1-day training session.

### Hours of Availability

CANCERLINE is currently available on the NLM computer from 5:00 AM Monday through 5:00 PM Saturday. Weekend service is also usually available through 11:00 PM Sunday.

### Charges

Searching will be billed on the basis of an algorithm that will include connect hour use, work performed by the NLM computer, and characters sent to the user's terminal. A 15-minute session may average about $5. Usage charges are $22/hour (10:00 AM–5:00 PM Eastern Standard Time) or $15/hour at other times during weekdays and all day on weekends. Each page of computer printout received in an offline print will cost $0.18.

### Subject Searching in CANCERLINE

CANCERLIT

To facilitate uniform, optimal retrieval, all CANCERLIT records added from 1980 forward contain the Medical Subject Headings (MeSH) developed and used by the National Library of Medicine. These headings should be entered exactly as they appear in MeSH and can be searched as they are searched in MEDLINE. Records entered in 1985 are also indexed with chemical names and CAS Registry/EC Numbers. Any significant word in the title or abstract fields can also be entered as a search term. All retrospective searches must use free-text, while 1980 searches can use both MeSH and free-text. Records input to CANCERLIT before 1980 are searchable by text words only.

CANCERPROJ

Records are searchable in CANCERPROJ by MeSH headings and chemical names/numbers as well as free-text searching. Additional controlled vocabulary terms (Keywords) are added to each project description for subject retrieval. These Keywords (KW) are directly searchable.

CLINPROT

Records in this file are retrievable by free-text searching. Also included are controlled vocabulary terms (Subject Captions) which are added to each protocol for subject retrieval. The Subject Captions field has an explode-like capability which can retrieve all levels of a selected hierarchy.

**Major Searchable Data Elements**

CANCERLIT

Title of the document
Abstract
Author
Journal name
Year of publication
Language of the document
Type of publication (journal article, technical report, etc)
Journal CODEN, Journal code or ISSN
MeSH headings of the National Library of Medicine (NLM)
Chemical name or CAS Registry/EC Number

CANCERPROJ

Project identification number
Title of the project
Abstract of project description
Keywords (controlled vocabulary indexing)
MeSH headings
Investigator
Organization performing research
Supporting agency
Initial and final year of project
Country where research is being performed
Chemical name or CAS Registry/EC Number

CLINPROT

Title of the protocol
Abstract (protocol entry criteria and objective)
Protocol details (outline, stratification points, study parameters, dosage schedule)
Subject and hierarchical codes (controlled vocabulary indexing)
Protocol chairman.
Organizations using the protocol
Protocol phase
Disease stage
Country and state where protocol is in use

## MEDLARS Centers

The organizations listed below are coordinating centers for all the MEDLARS centers in their geographic region. US centers can perform searches for you or identify a library closer to you that will do your searches on a "fee for services" basis. Non-US centers perform searches and also coordinate subscription services in their country for organizations that want to buy their own computer terminal and do their searches in-house.

US Centers

1. Greater Northeastern Regional
   Medical Library Program
   (CT, DE, MA, ME, NH, NJ, NY, PA, RI, VT, and Puerto Rico)
   The New York Academy of Medicine
   2 East 103rd Street
   New York, NY 10029
   Tel: (212) 876-8763

2. Southeastern/Atlantic Regional
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   Health Sciences Library
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   Baltimore, MD 21201
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   (IA, IL, IN, KY, MI, MN, ND, OH, SD, WI)
   University of Illinois at Chicago
   Library of the Health Sciences
   Health Sciences Center
   PO Box 7509
   Chicago, IL 60680
   Tel: (312) 996-2464

4. Midcontinental Regional Medical
   Library Program
   (CO, KS, MO, NE, UT, WY)
   University of Nebraska
   Medical Center Library
   42nd and Dewey Ave
   Omaha, NE 68105
   Tel: (402) 559-4326

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   5323 Harry Hines Blvd
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   Seattle, WA 98195
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   UCLA Biomedical Library
   Center for the Health Sciences
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   Canberra ACT 2600
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# ANNOUNCEMENTS

## Symposium on Pain Mechanisms and Management

***Maui, Hawaii, March 8–15, 1986***

The University of California at San Francisco (UCSF) is sponsoring this symposium on pain mechanisms and management to be held at the Maui Inter-Continental Hotel Wailea Resort. This symposium should be valuable to family practitioners, internists, rheumatologists, oncologists, neurologists, neurosurgeons, orthopedic surgeons, and others who treat significant numbers of patients with pain. This course will bring together UCSF and distinguished guest faculty to update participants on selected areas of research and on current approaches to common but difficult to treat pain problems.

Upon completion of this program participants will be able to: explain the anatomy and physiology of pain pathways and the brain mechanisms that inhibit these pathways; outline the structure, location, and function of endogenous opioid peptides; describe the pharmacokinetics of commonly used narcotic and non-narcotic drugs used to treat pain, recognize the indications for such drugs, the proper dose schedule and the optimal route of administration, list useful drug combinations and their common side effects, and safely withdraw patients from narcotic analgesics and manage withdrawal symptoms; understand the appropriate use of nonsteroidal anti-inflammatory drugs; manage chronic low back pain and obtain psychological evaluation of patients with chronic pain when necessary; list the common painful conditions produced by nerve damage; identify which painful conditions may respond to nerve blocks or ablative lesions of the nervous system, such as cordotomy; list common complications and side effects of such procedures; explain the mechanisms of cancer pain and multiple approaches to the treatment of cancer-related painful conditions; and understand the indications for and the use of devices designed for patient-controlled analgesia.

Registration will be limited, so early registration is advised. Date: Sunday, March 9, 1986; Time: 11:00 AM–4:00 PM; Place: Maui Inter-Continental Wailea, Hawaii. Fees: $400.00 for registration postmarked after December 15, 1984; $200.00 for a limited number of interns and residents (letter of verification must accompany registration to qualify).

Extended Programs in Medical Education of the University of California School of Medicine at San Francisco designates this continuing medical education activity as meeting the criteria for 23 credit hours in Category 1 of the Physicians' Recognition Award of the American Medical Association and the Certification Program of the California Medical Association. This program has been reviewed and is acceptable for 23 Prescribed Hours by the American Academy of Family Physicians. It has also been reviewed and approved by the American Pain Society. The California Board of Registered Nursing includes Category 1 courses of CMA-accredited continuing medical education to be acceptable toward meeting the continuing education requirements for license renewal. The American Nursing Association accepts AMA Category 1 hours as meeting the requirements toward recertification.

*For further information, contact:* Greenwood & Associates/MLH Travel-3505 Malina Pl, Kihei, Maui, HI 96743 (telephone: 800-262-6284; office hours: 8 AM–5 PM Monday–Friday, local time).

## Arizona Cancer Center 5th Winter Symposium on Advances in Clinical Oncology

***Snowbird, Utah, March 8–15, 1986***

*For further information, contact:* Mary Humphrey, conference coordinator, Arizona Cancer Center, Tucson, AZ 86724 (telephone: 602-626-6044).

## Functional and Stereotactic Neurosurgery

***Garmisch-Partenkirchen, West Germany, March 9–16, 1986***

The University of California at San Francisco is sponsoring a postgraduate course on functional and stereotactic neurosurgery to be held at the Holiday Inn in Garmisch-Partenkirchen, West Germany.

This symposium is designed to encourage the exchange of medical information on selected topics in functional and stereotactic neurosurgery between physicians from Europe and the United States. Topics to be discussed include: spinal cord stimulation for peripheral vascular diseases; implanted drug delivery system for the control of pain and spasticity; trigeminal ganglionic stimulation for the control of atypical facial pain; brain stimulation for the control of chronic pain; CT-guided stereotactic tumor biopsy and demonstration of BRW system; technique and results of stereotactic brain tumor irradiation by permanent radioactive nuclide implants and intracystic therapy; technique and results of stereotactic brain tumor irradiation by removable radioactive nuclide implants; and the effects of focal irradiation on normal brain. In addition to the didactic sessions, selected papers related to the topics will be presented by participants.

Submissions are welcome and should be sent to the Program Director.

*For further information, contact:* Extended Programs in Medical Education, University of California School of Medicine, Rm 569-U, San Francisco, CA 94143 (telephone: 415-666-4251).

### Counseling Strategies for Physicians

***Oahu, Hawaii, March 24-29, 1986***

The University of Texas at Tyler, in cooperation with The University of Texas Health Science Center at Houston, is sponsoring this meeting to introduce the latest techniques and research findings on matters that influence doctor-patient relationships. Faculty will discuss social change, family violence, addiction, and human sexuality as considerations in dealing with patients, and they will present problems likely to be encountered with specific groups of clients such as teenagers, minority groups, aging clients, and pregnant women.

The registration fee is $325; the program is approved for 17 hours in AMA/PRA Category 1 and is AAFP-prescribed.

*For further information, contact:* Division of Continuing Education, The University of Texas Health Science Center at Houston, 1100 Holcombe, Rm 1501, PO Box 20367, Houston, TX 77225 (telephone—within Texas: 1-800-392-4900-437, Program Code 975 DLB; outside Texas: 1-800-332-8747).

### Gastrointestinal Oncology—1986: An Advanced Postgraduate Course

***New York, New York, April 3-4, 1986***

The Continuing Medical Education Office at Memorial Sloan-Kettering Cancer Center is offering a course on gastrointestinal oncology. Directors of the course are Sidney J. Winawer, MD, and Robert C. Kurtz, MD. This program is aimed at physicians and surgeons who manage patients with gastrointestinal malignancies and gastrointestinal complications of other diseases including AIDS.

*For further information, contact:* CME Conference Planning Office, C-180, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021 (telephone: 212-794-6754).

### 1986 Annual Meeting of the American Radium Society

***San Francisco, California, April 6-10, 1986***

*For further information, contact:* Ms. Suzanne Bohn, Executive Secretary, American Radium Society, 925 Chestnut St, Philadelphia, PA 19107 (telephone: 215-574-3179).

### American Cancer Society National Conference on Urologic Cancer—1986

***Philadelphia, Pennsylvania, April 9-11, 1986***

The American Cancer Society is sponsoring this conference on urologic cancer to be held at the Adam's Mark Hotel in Philadelphia, PA. Topics included in the conference are: the role of staging in urologic cancer, integrated management of localized cancers, strategies for the management of recurrent and advanced cancers, and future developments and new research in genitourinary cancers.

The American Cancer Society is accredited by the Accreditation Council for Continuing Medical Education. This continuing medical education activity meets the criteria for 15 hours in Category 1 of the Physician's Recognition Award of the American Medical Association. Continuing education credits have been requested from the American Academy of Family Physicians and the American Osteopathic

Association.

The meeting will be open to primary care physicians, specialists in oncology, nurses, and students. Other interested health care professionals are welcome. Advance registration is requested. There will be an advance registration fee of $200.00 for physicians and dentists and $100.00 for all other health professionals. For registration received after April 1, 1986, the fee will be $250.00 for physicians and dentists and $150.00 for all other health professionals. A processing fee of $50.00 will be charged for cancellations after initial registration. Checks or money orders only will be accepted. There is no registration fee for students, interns, residents, and American Cancer Society Fellows or Career Development Awardees.

*For further information, contact:* the American Cancer Society, National Conference on Urologic Cancer—1986, 90 Park Ave, New York, NY 10016.

### First International Congress on Cancer and Hormones

***Rome, Italy, April 23-27, 1986***

This conference will include such topics as newer diagnostic and therapeutic approaches; epidemiology of endocrine-related cancer; hormone receptors and tumors; oncogenes, growth factors, and small effectors; hormones and cell proliferation; and cancers arising in endocrine glands.

*For further information, contact:* Stefano Iacobelli, MD, Laboratorio di Endocrinologia Molecolare, Istituto di Clinica Ostetrica e Ginecologica, Università Cattólica del S. Cuore, L.go Agostino Gemelli, 8, 00168 Rome, Italy [telephone: (06) 33.054.496; telex: 611330 UNCATRO].

### EORTC Symposium on GI Tract Cancer—Update on Combined Modality Therapy

***Heidelberg, Federal Republic of Germany, May 29-30, 1986***

The European Organization for Research on Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group is sponsoring this symposium which will focus on the role of perioperative and adjuvant therapeutic strategies in the treatment of cancer of the gastrointestinal tract. The experimental basis of multidisciplinary treatment will be explored and correlated with its latest application in various clinical settings involving primary, recurrent, or disseminated disease. Results of recent EORTC trials and state-of-the-art lectures will be presented. The topics of the meeting will be discussed in free papers and in poster sessions.

Free communications dealing with the topics of the symposium will be selected on the basis of submitted abstracts. Abstracts (in English) should be addressed to Professor Dr. P. Schlag, Chirurgische Klinik, Universität Heidelberg, Im Neuenheimer Feld 110, 6900 Heidelberg, Federal Republic of Germany, and should be received by *February 15, 1986.*

Seating is limited and preregistration is recommended. The registration fee is $120.00 US dollars (before March 1, 1986) and will be $150.00 US dollars thereafter.

*For further information, contact:* Dr. P. Schlag, Chirurgische Klinik, Universität Heidelberg, Im Neuenheimer Feld 110, 6900 Heidelberg, Federal Republic of Germany (telephone: 6221-56.21.11).

### Second International Symposium on Prostatic Cancer

***Paris, France, June 16-18, 1986***

The Second International Symposium on Prostatic Cancer will be held at the Intercontinental Hotel, 3, rue de Castiglione, Paris, France. Scientific contributions: poster presentations, and films, will be accepted. Category 1 and AUA Continuing Medical Education Credits will be awarded.

*For further information, contact:* Saad Khoury, MD, Clinique Urologique Hôpital de la Pitié, 83, Boulevard de l'Hôpital, 75634 Paris Cedex 13, France (telephone: 88-1-570.30.78); or James P. Karr, PhD, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263 (telephone: 716-845-2317).

### Sixth Annual Symposium on Fine Needle Aspiration

***Budapest, Hungary, Aug 9-16, 1986***

The Department of Pathology at the University of California School of Medicine in San Francisco and the Society of Hungarian Pathologists in cooperation with the California Society of Pathologists

are sponsoring the Sixth Annual Symposium on Fine Needle Aspiration, to be held at the Atrium Hyatt Hotel, Budapest, Hungary. This symposium is designed to provide a comprehensive introduction to fine needle aspiration biopsy techniques and interpretive criteria. Tuition includes lectures, extensive syllabus, Kodachrome study set, FNA performance, microscopes, microscopy sessions, and case presentations. The symposium offers ACCME/AMA/CMA Category 1 credits.

*For further information, contact:* Extended Programs in Medical Education, Rm 569-U, University of California School of Medicine, San Francisco, CA 94143 (telephone: 415-666-4251).

### Bone and Cancer: International Congress

***Hanasaari, Helsinki, Finland, October 9–11, 1986***

*For further information, contact:* European Forum for Orthopaedic Science Congress Bureau, PO Box 4, SF-00661 Helsinki, Finland.

### Third International Conference on Malignant Lymphoma: Current Status and Prospects

***Lugano, Switzerland, June 10–13, 1987***

The Palazzo dei Congressi is the location for this series of lectures and workshops dealing with the diagnosis and treatment of Hodgkin's and non-Hodgkin's lymphoma in adults and children. Most lectures will be given by invited speakers. The program will include free presentations, workshops, and a poster session. The deadline for the receipt of abstracts is *January 15, 1987.*

*For further information, contact:* F. Cavalli, MD, Division of Oncology, Ospedale San Giovanni, 6500 Bellinzona, Switzerland (telephone: 091-23-62-10).

## CORRECTION

The article "Phase I Study of Vinblastine and Verapamil Given by Concurrent Iv Infusion" by A. B. Benson, III, D. L. Trump, J. M. Koeller, M. I. Egorin, E. A. Olman, R. S. Witte, T. E. Davis, and D. C. Tormey (Cancer Treat Rep 69:795–799, July/August 1985) contains a mathematical error (*see* pages 795, 798, and 799). The mean vinblastine concentration of 2.2 ng/ml (correct) was incorrectly converted to 0.55 nM; the actual concentration is 2.7 nM. In addition, the mean verapamil concentration of 290 ng/ml (correct) was incorrectly converted to 0.45 μM; the actual concentration is 0.64 μM. The authors emphasize that this error does not alter their conclusions and that all other concentrations are appropriately presented.


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